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PHYSIOLOGICAL REVIEWS

VOL. 15 JULY, 1935 No. 3

OCCURRENCE, TRANSPORT AND REGULATION OF CALCIUM, MAGNESIUM AND PHOSPHORUS IN THE ANIMAL ORGANISM

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1. SOURCES, REQUIREMENTS AND ABSORPTION OF CALCIUM, MAGNESIUM AND PHOSPHORUS. The literature appertaining to the subject matter of this review has, in recent years, become so voluminous that a detailed survey cannot be given here. The attempt has therefore been made to point out certain lines of evidence which, to the writers, appear to stand out, keeping in mind, however, that as new experimental facts become available, many of these views will necessarily be subject to revision. This paper is an appraisal of the present status of the subject rather than the final word. A compilation of certain useful data taken from the literature has also been made. The bibliography is by no means complete. A sufficient number of references have been included, however, in order to permit those who may desire, either from the references given or through cross references, to obtain ready access to the literature. The references have been arbitrarily classified. Due to space limitation, no attempt has been made to place them under more than one caption, even though the subject matter of the article may warrant this.

a. Sources. Unless the elements included in the title to this review be administered in the form of added salts, the animal organism is dependent for its supply on the plant and it, in turn, derives its needs from the soluble constituents of the soil or, in the case of aquatic plants, from the surrounding aqueous media. Calcium and magnesium are present in sufficient quantity at all times in sea water to meet the requirements of plant life. However, this is not true of phosphorus. In many instances the available phosphorus may be the limiting factor. Soluble phosphates, chiefly in the form of calcium phosphates, are supplied to the sea either from the decomposition of animal and plant

life whose habitat is the ocean, or from the fresh water sources which pour the soluble extractives of the soil as well as suspended matter into the ocean. In regions where the terrestrial drainage is scant, and the supply via ocean currents is unfavorable, plant life and consequently animal life is less abundant.

Calcium and magnesium are widely scattered over the earth's crust, particularly in the form of carbonates, so that usually there are sufficient quantities of these elements present in the soil to supply the needs of the plant. Fresh water, unless derived from melting snow and stored in regions which are practically devoid of limestone, usually contain some soluble calcium and magnesium salts. It has recently been shown by C. M. McCay that trout and probably other fish may obtain a considerable proportion of their calcium directly from the surrounding water. In granite areas where the waters contain but little dissolved materials, the source of the mineral elements must be almost wholly dietary rather than from the aqueous environment. It has been pointed out by Orr that, in the Falkland Islands and in the hill pastures of Scotland and in other regions where the soil is chiefly siliceous with a resulting high acidity, there is a deficiency in the content of the alkaline earth elements in plants. Scarcity of phosphorus in soils may limit plant growth. Lack of phosphorus is, however, not as common a factor as scarcity of nitrogen. Plants with a low phosphorus content are widespread. Cattle maintained on the range grasses of western and middle western North America and in certain regions of South Africa, as has been shown by several groups of workers, exhibit marked symptoms of phosphorus deficiency. The low phosphorus content of these grasses may be due to *a*, low phosphorus content of the soil; *b*, non-availability as a result of extreme insolubility, especially in alkaline soils; *c*, lack of rainfall, and *d*, type of grass. Seasonal influence may be a factor of prime importance. Thus bur clover possesses relatively high nutritive value throughout the year. This is due to the fact that the burs which are relatively high in phosphorus are available to livestock even after they have matured and fallen to the ground. On the other hand, annual grasses and broadleaf alfilaria, while valuable early feed, have a comparatively short period when they furnish adequate nutrition. After the plant matures and dries, the seeds are not available, and the remaining leaves and stems are too low in minerals, especially phosphorus, to supply in the amounts which the animal can ingest the needs of growing livestock. The effect of rainfall may be in opposite directions depending on the season. It not only serves to effect solution of the soil constituents, but also to

bring to the surface mineral elements from lower strata. On the other hand, mature plants may suffer a decided decrease in mineral constituents, especially phosphorus, when exposed to the leaching action of late rains. The association of magnesium as an integral part of the chlorophyll molecule, combined with the smaller quantitative requirements of animal life and its wide distribution in the earth's crust, makes it improbable that under natural environment animal and plant life is seriously handicapped by lack of this element. It is more usual to find plants lacking the necessary chlorophyll as a result of iron deficiency rather than from a lack of magnesium.

Table 1, taken from the paper of Eckles, Gullickson, and Palmer, illustrates the range in the content of calcium, magnesium, and phosphorus in several of the common types of feed.

An excellent summary of the data relating to the mineral content of the common plants which serve as feed for western range cattle is given by Hart, Guilbert, and Goss. They record variations in the Ca:P ratio

TABLE 1

	CALCIUM	PHOSPHORUS	MAGNESIUM
Prairie hay	0.410–0.440	0.064–0.106	0.233–0.270
Timothy hay	0.179–0.395	0.112–0.144	0.102–0.197
Alfalfa hay	1.390–1.810	0.177–0.235	0.314–0.456

from unity to 39:1. In the latter case (dry leached broadleaf alfilaria) the percentage content of calcium was 1.17 and phosphorus 0.03. Their data show a variation in the calcium content of range grasses from 0.10 to 2.90, and in the phosphorus content from 0.03 to 0.68 per cent. The extreme ratios were found only late in the season.

While under natural conditions the limiting factor with respect to calcium, magnesium, and phosphorus for herbivorous animals is usually phosphorus, under artificial conditions, especially when animals are fed a ration which consists largely of grains and their by-products, the conditions may be reversed so that there is now a lack of calcium and an excess of phosphorus. This situation has been especially noted in attempts to bring hogs to quick marketable maturity.

The carnivorous animal finds greater difficulty in obtaining its calcium than its phosphorus requirements due to the fact that there is comparatively little calcium present in meat, whereas the phosphorus content is considerable. The calcium deficiency is, under natural con-

ditions, met by the ingestion of calcium phosphate which occurs in bones and cartilage, and, for the suckling animal, in milk. Some calcium may be ingested as dissolved calcium bicarbonate in the drinking water, especially in limestone regions.[1] The diet of the adult Eskimo is largely carnivorous. It includes much fish, sea animals, birds, and eggs. The children, however, are wholly or in part breast fed up to the age of 4 to 6 years. The omnivorous animal is more fortunate with respect to the list of calcium-containing foods which are available. Nuts and vegetables, and, above all, milk and its by-product, cheese, and eggs serve to supply calcium to the diet which otherwise, especially if it contains cereals and meat, would be rich in phosphorus and lacking in calcium.

TABLE 2

Approximate amounts of calcium and phosphorus in 100 grams of edible food

	CALCIUM	PHOSPHORUS	MAGNESIUM
	grams	*grams*	*grams*
Beef (lean)	0.007	0.218	0.024
Eggs	0.067	0.180	0.011
Egg yolk	0.137	0.524	0.016
Milk	0.120	0.093	0.012
Cheese	0.931	0.680	0.037
Wheat	0.045	0.423	0.133
Potatoes	0.014	0.058	0.028
Corn meal	0.018	0.190	0.084
Oranges	0.045	0.021	0.012
Almonds	0.239	0.465	0.251
Spinach	0.067	0.068	0.037
Beans (dried)	0.160	0.470	0.156
Linseed meal	0.413	0.741	0.432
Cottonseed meal	0.265	1.193	0.462

In many countries, especially those in which milk is used but little, beans form the staple food. They are fairly rich in calcium, magnesium, and phosphorus and, from the standpoint of supplying these elements, as well as protein, are, in many ways, comparable to milk. The calcium contained in soy bean curd is well utilized. Greater difficulty is experienced by the African native in obtaining the necessary intake of

[1] Water from such sources may contain 100 parts of calcium and 50 parts of magnesium per million. An animal drinking a liter of water per day would ingest 100 mgm. of calcium and 50 mgm. of magnesium. In certain regions this source may contribute considerably to the total daily intake of man as well as of native and farm animals.

calcium and magnesium. His diet consists in large part of maize which is low in these two elements but rich in phosphorus. Milk, butter, and eggs are seldom eaten. The children are usually breast fed up to 2 years. Mealie porridge is added as a supplement. In certain isolated communities, such as in Tristan da Cunha, the diet is high in carbohydrates, especially potato. This is, however, largely supplemented with milk, eggs, vegetables, and fish. The data in table 2, taken from Sherman's *Chemistry of Food and Nutrition*, are illustrative of the content of calcium and phosphorus in some of the common foods.

The importance of milk and milk products in the nutrition of the infant and the growing animal, as well as in the dietary of large masses of adult population, requires that special attention be given to this secretion as a source of the required alkaline earth elements and of phosphorus.[2] Furthermore, the drain on the maternal organism to supply these elements in the milk during the lactation period and the consequences that may result therefrom are matters of great importance. The latter aspects of this subject will be treated in another section. A summary of the data on the content of certain of the mineral constituents of human and of cow's milk is given in table 3. It is evident that human milk contains decidedly less calcium, magnesium, and phosphorus than cow's milk. The amount of magnesium, particularly in human milk, is quite small. This raises the question as to whether prolonged feeding solely on a milk diet might not lead to a deficiency with respect to this element. Instances are known where human infants have been breast fed up to six or more years of age. Cow's milk apparently supplies sufficient magnesium for the needs of rats during their life cycle, at least no difficulty has been reported when the mineral constituents were supplemented with iron, copper, manganese and iodine. Rats which are maintained on an exclusive milk diet, it is true, are prone to be sterile, but no evidence is available that lack of magnesium is the causative factor. No work has appeared in which milk was used exclusively to successfully rear a number of generations of animals.

Extensive studies on the various factors which influence human milk secretion have been carried out by Macy and her co-workers, Widdows,

[2] The daily ration allowed by law in the U. S. Navy includes 1 ounce evaporated milk or $\frac{1}{16}$ quart fresh milk. Some other dietary components allowed are: 1 pound hard bread (biscuits) or $1\frac{1}{4}$ pounds fresh bread; 1 pound tinned meat or its equivalent as fresh or salt meat, or 8 eggs; $1\frac{3}{4}$ pounds fresh vegetables or 3 gills beans or peas or $\frac{1}{2}$ pound rice or other cereal; $\frac{3}{16}$ pound dried fruit or $\frac{3}{8}$ pound tinned fruit. For complete list see article 1320, sec. 13, U. S. Navy Regulations.

TABLE 3

The content and distribution of the alkali earth elements and of phosphorus in human and cow's milk

SUBSTANCE	NUMBER OF OBSERVATIONS	RANGE*	MEAN*	SOURCE OF DATA
Human milk				
Total calcium	36	22.5–42.8	30.0	Telfer
Diffusible calcium	8	14.4–21.8	18.0	Hess and Benjamin
Magnesium	36	2.5– 5.5	3.5	Telfer
Total phosphorus composite		16 –18.5	17.5	Widdows, Lowenfeld, Bond and Taylor
Total phosphorus composite†			21.0	Telfer
Total phosphorus composite‡			13.5	Telfer
Inorganic phosphorus	8	3.6– 8.4	6.1	Hess and Benjamin
Diffusible phosphorus	8	2.5– 6.1	3.8	Hess and Benjamin
Iron			0.1	Telfer
Casein per cent			0.8	Abderhalden
Albumin per cent			1.2	Abderhalden
Milk sugar per cent			6.4	Abderhalden
Cow's milk				
Total calcium	40	110–145	128	Sanders
Diffusible calcium	21	33– 44	39	Hess and Benjamin; Sanders
Magnesium	13	13– 17	15	Sanders
Total phosphorus	31	90–130	104	Mattick; Sanders
Acid soluble phosphorus	18	60– 95	79	Sanders
Inorganic phosphorus	10	66– 80	72	Hess and Benjamin
Diffusible phosphorus	10	29– 39	35.5	Hess and Benjamin
Iron			0.08	Telfer
Casein per cent	7	2– 3	2.5	Sanders
Albumin per cent			0.5	Abderhalden
Milk sugar per cent			5.0	Abderhalden

* Minerals are expressed in milligrams of the element per 100 ml. of milk.

† Milk from healthy country women.

‡ Milk from ill nourished women of city slums.

TELFER, S. V. Glasgow Med. J. **113:** 246, 1930.

HESS, A. F. AND H. R. BENJAMIN. Proc. Soc. Exp. Biol. Med. **30:** 1358, 1933.

WIDDOWS, S. T., M. F. LOWENFELD, M. BOND AND E. I. TAYLOR. Biochem. J. **24:** 326, 1930.

ABDERHALDEN, E. Textbook of physiological chemistry. New York, 1911, p. 654.

SANDERS, G. P. J. Biol. Chem. **30:** 747, 1931.

MATTICK, E. C. V. Biochem. J. **22:** 144, 1928.

Lowenfeld, Bond, and Taylor, as well as many others. Extensive bibliographic references to the literature are given in their publications. It appears that there are individual differences in the mineral constituents of human milk which are greater than the daily variation in the same individual. The secretion by the two mammae are quite uniform in their constituents including calcium and phosphorus. Total solids, phosphorus as well as fat and proteins, tend to increase with time during each nursing period. The percentage of calcium and phosphorus secreted in the milk varies during the 24-hour period in each individual; the rhythm is not, however, the same for all individuals. Studies which were carried out over the entire lactation period showed that there was a gradual decrease in the concentration of ash and of calcium; the phosphorus also gradually decreased, but increased during the latter part of the lactation period along with fat and total solids. The constituents in the milk of neurotic individuals appear to be more variable than in the case of those whose temperament is more phlegmatic. Nervous influences resulting from the collection of excreta during metabolic experiments may influence calcium absorption in animals. Constituents of the diet, especially the vitamins, do not appreciably influence the mineral content of human milk. The first effects of an inadequate diet are on the quantity of the milk secreted rather than on the non-lipoidal constituents. It is probable, however, that, as a result of prolonged inanition, the chemical composition of milk is materially influenced. The daily variation, as well as the variation in milk composition over the entire period of lactation, probably has but comparatively little influence on the nourishment of the infant since, except for those constituents in which milk is decidedly deficient, any lack in quality may, within certain limits, be compensated by increased ingestion.

Attempts have been made to correlate the content of calcium and phosphorus in milk with the incidence of rickets. However, the modern work on the etiology of rickets, together with the fact that with the proper vitamin supplements, rachitic-free children may be reared on milks which vary as widely as human, cow's and goat's milk, lends but little support to such a theory. Breast milk is a poor source of vitamin D and hence both breast fed and artificially fed infants are accordingly subject to rickets unless the diet is supplemented with this vitamin. The calcium and phosphorus in human milk are somewhat better utilized by the infant than when taken in cow's milk. This may possibly be related to such factors as fat content, the amount of sodium and potassium chloride, content of casein, etc. However, there is ample evidence

that cow's milk may supply the mineral needs of the human infant, at least for a period of some months following birth.

An interesting generalization, attributed to Abderhalden, is that protein, calcium, and phosphorus are contained in the largest quantity in the milks of rapidly growing animals (rabbit, dog, cat), less in the milk of the slower growing animals (cow, sheep, goat), and least in such slow growing animals as the human. For example, the calf doubles its body weight in 47 days following birth while it requires 180 days for the human infant to accomplish the same thing. The ratio of the mineral constituents between cow's and human milk is about the same (4:1). No such strict arithmetical relationship holds for all species of animals. The low concentration of minerals in human milk implies, as is actually found, a relatively small daily requirement of the bone forming elements in early infancy in accordance with the slow rate of skeletal growth and the long period of early development of the human infant. It would appear then that undiluted cow's milk is unnecessarily rich in the mineral constituents when used for infant feeding; on the other hand, it affords for the older child and adult man a most valuable supplementary source for calcium and phosphorus.

Another interesting relationship is the approximately constant proportion of calcium, phosphorus and magnesium in milk from different species. Telfer has suggested that this is related to the uniformity in the mineral composition of bone in all animal species having a like skeletal system. This relationship does not hold, for instance, with respect to the iron content of milk which, in spite of differences in the percentage content of the other elements, is about the same in both cow's and in human milk. This is a factor which warrants serious consideration since, in diluting cow's milk for infant feeding, the content of iron is reduced far below the amount contained in human milk. Fortunately, nature has wisely provided sufficient reserve iron in the body at birth. However, a prolonged milk diet without added iron-containing supplements, will inevitably lead to an anemia.

Telfer has also calculated the human milk requirement and the minerals thus ingested during infancy. He cites 120 calories per kilo of body weight per day as the requirements for maintenance and growth during the 6 months period following birth. Since the fat, protein, and carbohydrate content of human milk yields 736 calories per liter, the daily milk intake is approximately 0.165 liter per kilo of body weight. On the same basis, the approximate intake of calcium, phosphorus, and magnesium is respectively 50, 45 and 10 mgm.

Reference to table 3 shows that about two-thirds of the calcium in human milk is diffusible, while only about one-third is similarly present in cow's milk. The non-diffusible portion presumably exists partly in combination with the casein and other proteins present in milk and partly as colloidal calcium phosphate. Evidence for the presence of colloidal calcium phosphate is based on the proportion of ultrafiltrable phosphorus. According to Hess and Benjamin, human milk contains about 62 per cent and cow's milk about 50 per cent of diffusible inorganic phosphorus. Numerically, the difference between human and cow's milk is much greater since in the former only about 6 mgm. per cent of the phosphorus or about one-third is inorganic, while an average of 72 mgm. per cent or nearly three-fourths of the total is so present in cow's milk. It is unlikely, according to Hess and Benjamin, that the differences in composition between human and cow's milk are directly responsible for the greater efficacy of human milk in the prevention of rickets. The high content of lactose in milk is favorable to the growth of acid-producing bacteria. The production of lactic acid in the intestinal tract favors solution and probably also absorption of calcium phosphate as well as other salts of calcium and also of magnesium.

b. Mode of combination. The form in which the three elements under consideration occur in natural foodstuffs is in part determined by the reaction. It is very probable that some of the ash is present in the form of inorganic salts and particularly as calcium phosphate. In milk the amount of calcium or calcium phosphate is considerable. An acid reaction tends to promote solubility, while an alkaline medium favors insolubility. Phosphorus always occurs in biological material in the oxidized form, namely, as derivatives of phosphoric acid. It may be present in animal or plant tissues as an inorganic salt, usually calcium phosphate, as lecithin or other phospholipoids, nucleic acid or nucleoprotein, or as phosphoprotein in which the phosphoric acid, as shown by the work of Rimington and of Levene, is also in the form of an ester, and particularly as serine phosphoric acid. From the standpoint of absorption and utilization, the mode in which phosphoric acid is combined is important. If it be combined as an ester it is subject to hydrolytic cleavage in the alimentary tract of the animal. Time is a factor of great importance in this connection. It delays the absorption of phosphoric acid so that in all probability much of the calcium is absorbed before most of the phosphoric acid is set free. This is a wise provision of nature since otherwise a considerable amount of calcium phosphate would be formed and this, having a somewhat low solubility,

would probably lead to a decreased absorption of both elements. It has been amply demonstrated that the amount of calcium which is absorbed is considerably reduced when a soluble salt of phosphoric acid is administered. As has already been stated, some of the magnesium occurs in the plant in the form of chlorophyll. Probably as the result of bacterial action, and of alimentary enzymes, and particularly of the hydrogen ion of the gastric juice, magnesium is set free as an inorganic salt and is absorbed as such. The form of the inorganic phosphoric acid-containing compounds of magnesium is, like calcium, determined in part by the reaction of the media. A characteristic property of the alkaline earth elements, as has been shown, particularly by the work of Greenberg and Schmidt, Kirk and Schmidt, and Miyamoto and Schmidt, is to form complex ions with proteins. Miyamoto and Schmidt attribute the formation of these complex ions to the combination of the alkaline earth element with the carboxyl groups of the protein molecule. The presence of such complexes can be demonstrated on passing an electric current through a solution of protein dissolved in an alkaline earth hydroxide. Due to step dissociation, some of the alkaline earth element remains combined with the protein molecule and, in migrating, under the influence of an electrical current, acts in this respect like a complex ion. These facts are important in explaining the mode in which calcium and magnesium are held in solution in many of the biological fluids, especially milk and blood serum. It is in the form of such protein complexes that the non-diffusible calcium of normal blood occurs. Calcium and magnesium are present, particularly in the plant juices, as the salts of such weak acids as carbonic, lactic, citric, tartaric, and malic, and as calcium phosphate. Such salts are probably but little dissociated when in combination with these acids. In sea water, calcium and magnesium are found chiefly as chlorides.[3]

c. Quantitative requirements. It is obvious that the quantitative requirements of the animal organism for calcium, magnesium, and phosphorus will depend in part on its state of nutrition and more particularly on whether it has reached the adult stage or whether it is still growing, on whether it is pregnant or lactating, or, in the case of the hen, on whether or not it is laying. The bulk of the calcium and magnesium found in the animal organism occurs in the skeleton. There is comparatively little calcium present in the soft tissues and in the body fluids. The soft tissues contain more magnesium than calcium.

[3] The average composition (mgm. per kilo) of sea water is: Na, 11000; K, 400; Ca, 400; Mg, 1300; Cl, 19000; SO_4, 2700; CO_2, 80–90; B, 4–5.

On the other hand, phosphorus is present not only in the skeleton, but is also widely distributed in the soft tissues and in the body fluids. In order to understand its metabolism, consideration must be given to the twofold functions which are assigned to phosphorus.

It has come to be generally recognized that the skeleton is not a rigorously fixed system, but rather a mobile one in which there is both influx and outpouring of inorganic constituents. The skeleton constitutes a great reservoir from which the body can very largely, in time of need, withdraw the three elements in question. Aub and his coworkers have shown that the trabeculae of the long bones constitute the seat of readily mobilizable calcium. The withdrawal is, however, not limited by the amount stored in the trabeculae. Calcium may also be withdrawn from the shafts of the long bones. Replacement takes place subsequently when the intake is adequate. In experiments which have not yet been published, Goss was able, by placing rats on a low calcium diet and subjecting them to repeated periods of pregnancy and lactation, to withdraw about 60 to 70 per cent of the body's total store of calcium. Clark has recorded data showing that certain of the inmates of San Quentin prison in California stored marked amounts of calcium and phosphorus. They had, prior to their incarceration, apparently subsisted on haphazard diets which were particularly low in calcium. Others whose diets had been adequate, stored but little or none. Lack of calcium is usually characteristic of prison diets. It seems altogether probable that the skeleton, as well as the soft tissues, has a considerable endogenous metabolism of calcium, magnesium, and phosphorus. Even in periods of inanition, the amounts which are excreted are considerable. This fact stands in strong contrast to the excretion of sodium, potassium, and chlorine which, in continued periods of inanition, practically ceases. The source for this endogenous output of the three elements is in large part the skeleton. However, the soft tissues may contribute considerably to the endogenous output of phosphorus. In order to maintain the status quo of the body, these elements must be supplied at least in amounts which equal those lost in the processes incident to endogenous metabolism.

Prior to birth, the fetus must obtain its requirements of the mineral elements from the mother. They are supplied either from the amounts which are present in the mother's diet, or, if that be insufficient, by withdrawal from the skeleton and the tissues. The quantitative need for calcium, phosphorus, and magnesium depends on the age of the fetus. It is obvious that the demands of the soft tissues will precede

those of the skeleton. Widdows has cited data which illustrate the calcium requirements of a human fetus. For the first four months of fetal life, the daily calcium requirements were 3.4 mgm., for the next month 59 mgm., for the fifth to the seventh month period 62 mgm., for the eighth month 60 mgm., and for the ninth month 455 mgm. The phosphorus requirements will not be strictly parallel to these figures since the fetus at an early age needs phosphorus for its soft tissues. As will be shown in greater detail later, during periods of heavy demand upon the mother as in pregnancy and, more especially, during lactation, the animal may be in negative balance. This is the result of the fact that the rate of absorption of calcium and of phosphorus is not sufficiently rapid to meet the needs of the various channels of excretion, especially milk. A high egg producing hen may likewise be in negative calcium and phosphorus balance. Ample provision is made in the content of calcium, magnesium, and phosphorus in the egg to meet the requirements of the embryonic chick.

During the period of growth the demands of the body for the three elements are especially heavy and it is at this time that dietary deficiencies particularly manifest themselves. Heifers maintained on the range grasses which are exceptionally low in phosphorus (0.1 per cent or less of P and a Ca/P ratio of 12/1 with abundant vitamin D) will exhibit after a period of 5 to 7 months a condition which is known as styfsickte or stiff sickness. The condition is characterized by retardation of growth, abnormal skeletal development, and thickening of the epiphysis of the long bones. The gait of the animal is stiff and lame. Such animals show an abnormal craving for substances other than the natural feed. This is manifested by coprophagy and bone chewing. The condition of aphosphorosis has been studied extensively in America by Eckles, Gullickson and Palmer, and by Guilbert and Hart, and in Africa by Theiler and Green. Extensive literature references are contained in their publications. The plasma inorganic phosphorus may fall as low as 1 mgm. per cent, while the blood calcium remains normal. Administration of inorganic phosphates such as $Ca_3(PO_4)_2$ or NaH_2PO_4 or of phosphorus-rich foods usually leads to a marked gain in weight and the restoration of the inorganic plasma phosphorus to a level of 5 to 7 mgm. per cent. The level of milk production is decidedly decreased as a result of phosphorus deficiency. There is usually an appreciable reduction of ash in the bones which is replaced by water, lipid, and other organic substances. The dry fat-free bones (femur, humerus, ribs) are characterized by a low total ash and a lower $Ca_3(PO_4)_2$, and a higher

$CaCO_3$ content than normal bones. The result is a low calcium phosphate to calcium carbonate ratio, a characteristic of rickets in laboratory animals. Guilbert and Hart tested the effects of different calcium and phosphorus intakes on the estrous cycle and reproduction of rats. They conclude that diets containing 0.18 to 0.22 per cent of phosphorus with a Ca/P ratio of approximately 4:1, when fed to rats during the period from sexual maturity to about 100 days of age, caused ovulation to be irregular or to cease entirely. This level of phosphorus, however, appeared to be adequate to maintain normal estrous cycles in rats which were about 110 days of age. However, their diets may have been deficient in other respects. The body cannot make extensive demands for phosphorus upon its skeletal system in order to supply its tissue needs. There appears to be no mechanism for dissociating the phosphoric acid from its combination with calcium and retaining the former while the latter is eliminated.

Calcium deficiency manifests itself in young animals by a decreased rate or a cessation in growth. Calves show an unthrifty appearance. The plasma calcium may be low. The animals exhibit a craving for dirt, cinders, or boiler scale. There may be sudden collapse. Lack of calcium in pigs, especially when the animals are on a high phosphorus diet, leads to a paralytic condition of the fore or hind legs. Plasma calcium, especially the diffusible, is markedly decreased. Mature animals on a calcium deficient diet will reproduce, drawing largely upon their skeleton for the necessary calcium and phosphorus and, to a lesser extent, upon the soft tissues for phosphorus. There is no serious interference with the milk production until the calcium losses exceed about 20 per cent of the animal's total supply. Meigs and his co-workers state that the total calcium loss of a cow usually does not exceed 20 per cent. The nutritive disasters which they observed on low-calcium rations were due to low content of vitamin A rather than of calcium. Under natural conditions there appear to be no reports dealing with magnesium deficiency in cattle. In most cases this condition would probably be associated with a deficiency in calcium or, if not, it would be mistaken for the latter condition. McCollum and his associates have carried on experiments to elucidate the question of magnesium deficiency in rats. On a ration of about 0.2 mgm. per 100 grams of food, the animals presented a symptom complex which was characterized by vasodilatation, hyperirritability of the nervous system, cardiac arrhythmia, tonic-colonic convulsions, and finally, death. The syndrome of magnesium deficiency constitutes, in their estimation, a form of tetany.

In children, lack of calcium, phosphorus (or of both) or vitamin D in the diet leads, as in other vertebrate animals, to the condition which has come under the general term of rickets. It is most common when children are subjected to an enforced unbalanced dietary regime as in famine or war. Even in communities where the food supply is presumably plentiful, cases of rickets still occur. More often these are due to lack of vitamin D or of sunshine rather than lack of the necessary mineral elements. Three types of rickets are now distinguished.[4] In the first the calcium content of the serum is normal, but the phosphorus content is low. This leads to overgrowth of the osteoid tissue at the ends of the long bones and at the rib junctions. Due to retarded calcification, the bones fail to acquire normal rigidity and as a result of pressure of the weight of the body, the ends tend to enlarge. When the calcium content, particularly the diffusible fraction, is low, and the phosphorus content is normal, tetany results. Bone growth stops and resorption of tissue may occur so that the need for calcium and phosphorus may be reduced. When both serum calcium and phosphorus are reduced, bone growth also ceases, but the histological picture differs somewhat from each of the two previously mentioned cases. Osteomalacia, or withdrawal of the mineral elements from the skeleton, may result when a population is subjected to famine, or is forced to subsist on diets which are notoriously deficient in the essential mineral elements. The amount of decalcification depends, of course, on the severity of the famine, the duration, and, in the female, on whether or not lactation occurs. In sheep, extensive decalcification has been noted when the animals were grazed on feed which contained oxalic acid.

The optimum intake of the three elements under consideration for the common animals is not known with certainty. As has been stated before, this will depend on whether the animal is growing or mature, or whether it is pregnant or lactating. The data of Goss and Schmidt show that the daily amount of calcium and phosphorus required to maintain a normal rat in balance is about 90 to 100 mgm. and 60 to 80 mgm. respectively. During pregnancy these figures are increased about 10 to 20 per cent, while during the lactation period, the input of calcium and of phosphorus respectively may be as high as 225 and 150 mgm. During growth, the daily input of calcium and phosphorus for a 20 to 30 day old rat was found to be about 60 to 90 mgm. and 45 to 55 mgm. respectively. At 60 days these figures rose respectively to about 175 and 150 mgm.

[4] This classification is somewhat arbitrary.

It is significant, as Sherman and Quinn point out, that, while the body of the rat in making a normal growth and development from birth to maturity, multiplies its original weight by about 70, it must multiply its original body phosphorus by about 150 and its calcium by about 340. Hart, Steenbock, and their associates estimate that a lactating cow should receive at least 4 grams of CaO per pound of milk production with a somewhat safer figure at a level of 6 to 8 grams. The requirements for P_2O_5 are placed at the same figure. In 1920 Hart and his associates estimated that the ration of an herbivorous animal should contain at least 0.45 per cent of calcium oxide calculated on the basis of the total ration. Palmer and his associates at the University of Minnesota maintained cattle for a period of 3 years on a ration which contained 0.18 per cent of Ca on the basis of dry weight and found no lessened breeding efficiency. Normal breeding was also shown in two other groups maintained on rations which contained respectively 0.32 and 0.64 per cent of calcium. The animals were not high milk producers. All of the cows were, however, in negative calcium and phosphorus balance. The relation of input of calcium and of phosphorus to output during pregnancy and lactation will be discussed in another section.

Sherman has carried out extensive studies to determine the normal calcium and phosphorus requirements of man. In 97 experiments he found daily calcium outputs which ranged from 0.27 to 0.82 gram with an average value of 0.45 gram. Allowing a 50 per cent safety margin, the dietary standard becomes 0.68 gram. Perhaps a rounded figure of 1 gram per day would include an ample margin of safety for an average population. The daily phosphorus requirements per 70 kilos of body weight varied from 0.52 to 1.20 gram with an average of 0.88 gram. A daily input of 1.0 to 1.2 gram is probably a safe figure for an average population. The phosphorus requirement is about 1/40 to 1/50 of that of the body's need for protein when the diet is not a restricted one. The needs of the growing child for supplementary foods containing calcium and phosphorus are illustrated by the experiments of Sherman and Hawley. Children of 3 to 13 years of age on a mixed diet which was supplemented daily for each child with 750 grams of milk stored 0.15 to 0.62 gram of calcim per day, or on the average, 10 mgm. of calcium and 8 mgm. of phosphorus per kilo body weight per day. When the milk allowance was increased to a liter of milk per day, the average daily storage, expressed in the same units of calcium, increased to 17 mgm. They regard a liter of milk per day as a necessary supplement to the mixed diet of a growing child. This diet would contain at least

1 gram of Ca and a proportionate amount of phosphorus. Comparisons of the growth requirements of different animals cannot be strictly made since such data would necessarily take into account the rate of growth and the activity. For farm animals whose activity is restricted, the latter factor is especially important.

There appears to be no correlation between the monthly cycle of women and the demands for calcium and phosphorus. Experiments designed to determine the needs of the human body during pregnancy and lactation have yielded less conclusive data than similar experiments carried out on animals. This is due to the restricted number of such experiments, and the inability of most experimenters to follow the balances during the entire period of pregnancy and lactation. However, it is obvious that fetal storage of the three elements must take place. The work of Macy, Hunscher and their collaborators indicates that in pregnancy there may be a loss of calcium with a simultaneous storage of phosphorus. It is not clear what factors operated to bring about these divergent results. In spite of large intakes, loss of calcium was noted during the period of heavy lactation. Coons and Hunscher estimate that pregnant women store about 200 mgm. of calcium daily from the 4th to the 9th month of pregnancy, and 300 mgm. daily during the last week of pregnancy. The daily phosphorus retention is about 150 to 200 mgm. by the 5th month and 200 to 300 mgm. for the remainder of the pregnancy.

It is evident that when the intake of protein, particularly neucleoprotein, is high, the needs of the human body for phosphorus will be easily met. However, the intake of phosphorus in dietaries upon which a not inconsiderable proportion of the population of the southern states in the United States subsists, and which consist in large part of patent flour, degermed corn meal, sugar, and fats, may fall below the desirable level despite an adequate intake of protein unless the diet is supplemented with eggs and milk. The calcium situation is not so fortunate. The high American meat-containing diet provides but little calcium. The high percentage of purified foods such as sugar and fats helps to reduce the input. It is probable that the average mixed diet is more deficient in calcium than in any other element. The inability of certain individuals to tolerate the large bulk which is necessarily associated with a vegetarian diet prevents the more general use of these foods for purposes of providing the mineral supplements. It is evident, therefore, that man must necessarily look to the dairy products and to eggs for the needed calcium supply unless he takes them in the form of added mineral

salts. It should be pointed out that inability to tolerate the amount of fat contained in whole milk may necessitate the use of skimmed milk by certain individuals. It does not appear probable that the average mixed diet is deficient in magnesium especially if it includes the leafy vegetables. It is noteworthy, however, that milk and eggs are particularly low in magnesium (see tables 2 and 3).

A comparison between the Finnish and the American diet (see table 4) indicates that such factors as dairy products operate to maintain the former diet at a level which is considerably higher in calcium, magnesium, and phosphorus than is shown by the American diet.

d. Availability of calcium, magnesium and phosphorus in various diets. Since, in the course of digestion, the calcium, magnesium and phos-

TABLE 4

*Daily input of P_2O_5, CaO and MgO in certain elective diets**

CALORIES	FINLAND				U. S. A.			
	P_2O_5	CaO	MgO	No. of cases	P_2O_5	CaO	MgO	No. of cases
	grams	*grams*	*grams*		*grams*	*grams*	*grams*	
> 4,000	10.86	6.10	2.02	7	4.24	0.79	0.89	3
3,500–4,000	9.46	3.79	1.85	6	3.22	0.94	0.51	3
3,000–3,500	8.18	4.02	1.53	10	3.29	0.99	0.50	6
2,500–3,000	6.93	3.51	1.23	10	3.20	0.92	0.46	3
2,000–2,500	5.64	2.96	1.03	17	2.06	0.36	0.32	4
1,500–2,000	5.12	2.85	0.78	16	1.84	0.68	0.23	1
1,000–1,500	3.36	2.34	0.51	4	—	—	—	—
< 1,000	2.44	2.21	0.37	2	—	—	—	—

* TIGERSTEDT, C. Skand. Arch. Physiol. **56**: 265, 1929.

phorus which are contained in most foodstuffs are set free in the form of inorganic salts, it should follow that the form in which these elements are ingested is not material. Mellanby and others have shown that cereals possess an "anticalcifying" action. This appears to be due to the presence of inositol phosphoric acid which is not easily split or absorbed and hence is probably not available to the extent that it would be if it were present in other forms. It is essential for the purpose of absorption that the salts be soluble. A number of experiments, whose object was to determine the availability of the three elements in various foods, have been carried out. Sherman and Hawley found that the calcium of milk was used more efficiently by children than the calcium of vegetables. This is not unexpected, since it is probable that the

vegetables were not thoroughly masticated and a part at least escaped digestion with the result that some of the mineral elements were excreted with the undigested residues. Blatherwick and Long believe that calcium and phosphorus derived from vegetables are capable of meeting the requirements of man. Other workers have obtained data in agreement with this. These facts should not be taken to mean that vegetables may be generally used in place of the more concentrated types of calcium-containing foods since many children and adults with low intestinal gradients are unable to tolerate the bulk which is necessarily associated with an adequacy in the intake of these mineral elements. There appears to be no essential difference in the availability for rats between the lactate, carbonate, sulfate, phosphate, and silicate salts. Greenberg and Gunther found that the increase in serum calcium was essentially the same irrespective of whether calcium chloride, gluconate, lactate, or acetate was ingested. The inference is that weak organic salts are converted into the chloride in the stomach by the hydrochloric acid of the gastric juice. In this connection it should be pointed out that calcium chloride may serve as an important source for the chloride ion. The ingestion of sweet milk by human subjects did not lead to an increase in the blood calcium, but the inorganic serum phosphate did increase. On the other hand, the ingestion of acidified milk resulted in a marked increase in both blood calcium and phosphorus. These data do not necessarily indicate a difference in the availability of calcium and phosphorus in the two types of milk, but rather a difference in the rate of absorption which is favored by the presence of acid in the milk. Calcium carbonate and gluconate are utilized equally well by the growing chick, but the gluconate functions slightly more efficiently than the carbonate in the laying hen. There is no essential difference between fresh and evaporated milk with respect to the availability of its salts. However, judgment must be exercised when it is desired to increase the input of calcium by not adding calcium in the form of a phosphoric acid salt to a ration of high phosphorus content. Likewise, it would be unwise to add calcium fluoride. However, any palatable calcium salt of a weak acid will probably serve to bring calcium-deficient rations to a level which will meet the requirements for growth and maintenance.

The carthartic effect of certain magnesium salts probably hinders their complete absorption when large doses are ingested. However, the work of Winter and Richey indicates that relatively large amounts of magnesium can be absorbed, and that this takes place in the stomach.

Vomiting produced by magnesium chloride was found to be associated with increased magnesium absorption into the blood stream in spite of loss by the mouth. A high content of magnesium in the diet may decrease the assimilation of calcium and phosphorus, especially, as Haag and Palmer have pointed out, when the amount of the latter contained in the diet is low. Elmslie and Steenbock found that excessive amounts of magnesium salts given to rats rendered the diet unpalatable, with a resultant lowered intake of food. Digestive disturbances followed. Lesser amounts were without influence on calcium metabolism. The severity of rickets in rats (high calcium and low phosphorus) was not influenced by added magnesium salts. Huffman and his co-workers showed that the inclusion of 3 to 5 per cent of magnesium salts in the rations of cows maintained on a low calcium diet had no deleterious effect nor did it affect the retention of calcium and phosphorus. The irritability which was shown by a calf raised on an exclusive milk diet was relieved by the administration of magnesium phosphate. Carswell and Winter found that approximately half of an 8-gram dose of magnesium lactate was absorbed. They believe that with an adequate intake of phosphorus, magnesium favors calcium storage, but how this is brought about is not definitely stated. The work of Barbour and Winter on dogs indicates that an adequate intake of phosphorus is essential to prevent losses of calcium on administration of magnesium salts. In fact, adequate phosphorus intake is regarded by them as serving to build up the body's reserve of both calcium and magnesium. Extra calcium was retained when the P_2O_5 intake exceeded 4 millimols per kilo per day. While, from an experimental standpoint, the above facts regarding magnesium are interesting, they probably play no important rôles in ordinary mixed diets or rations.

The content of magnesium in the bones of rachitic animals is greater than in the bones of normal animals. Thus von Euler and Rydbom state that normal bones contain 24.4 parts of calcium and 0.10 part of magnesium, while the figures for the corresponding elements in the bones of rachitic animals are respectively 21.5 and 0.63. This is to be expected since rachitic bones contain a relatively larger percentage of soft tissue which is high in magnesium. The bones of fishes contain relatively more magnesium (42:1) than the bones of warm blooded animals. They are also less hard due probably to a greater content of organic matter. The specific rôles played by magnesium in life processes require special mention. It acts as an antagonist to calcium. It activates phosphatase, and it is concerned in carbohydrate metabolism. There is more

magnesium than calcium present in muscular tissue. The Mg/Ca ratio in the tissues of warm blooded animals ranges from 10:4 to 10:1, while in the soft tissues of the fish the ratio is 1:2. The minimum daily intake of magnesium for rats has been estimated to be about a milligram. Lavollay and von Euler and Virgin obtained maximum growth in rats when the dry feed contained 30 to 50 mgm. of magnesium per 100 grams of food. The magnesium requirements for an adult man have been set at 600 mgm. per day. Further work, however, should be carried out to determine more precisely the magnesium requirements for various animals.

e. Factors which influence the absorption of calcium, phosphorus and magnesium. As has been stated before, the function of the alimentary tract is to bring the above named elements into a condition so that they can be absorbed. The chief agencies to be looked for are the enzymes which accelerate the splitting of phosphoric acid from its ester compounds, and hydrogen ions which are instrumental in keeping calcium phosphate in solution. Neither of these agencies operates to any extent in the mouth. Becks considers pH = 7.15 as the average normal reaction of human saliva. Acidities of pH 6.5 have been reported. It is very probable, as a result of bacterial action, that considerable variations on either side of these figures may occur in isolated portions of the mouth. The data of Clark and Levine point to such a possibility. According to Becks, the average content of calcium, magnesium, and phosphorus, expressed as milligrams per cent, is respectively 12.1, 1.3 and 4.3. Variations in the calcium content, ranging from 6 to 14 mgm., and in phosphorus from 4.0 to 6.0 mgm., have been reported. The mineral content of saliva is subject to variation during the day. A portion of the calcium is probably combined with mucin to form a calcium-protein complex. According to Becks, the concentration of $CaCO_3$ and of $Ca_3(PO_4)_2$ in saliva is not such as to form a saturated solution. It is altogether probable that, partly as a result of the escape of carbon dioxide and the formation of ammonia by bacteria, local zones of alkalinity are produced which favor deposition on the teeth of a complex mixture of calcium-containing compounds in the form of "tartar." Attempts to correlate the mineral content of saliva with dental caries have shown that, in the latter condition, there are no outstanding variations from the normal. Becks has published figures which show a decrease in calcium, magnesium, and phosphorus in the majority of patients affected with pyorrhea. In pregnancy, magnesium is increased and calcium is decidedly reduced. The significance of these data is not

apparent. The chief function of the process of mastication is to break up the particles of food. This leads to the discharge of some of the soluble salts contained in the tissue cells into the saliva. In those animals which swallow the food without previous mastication, this factor does not enter. In birds, the process of breaking up the solid food particles is accomplished in the gizzard. Both as a result of bacterial action in the rumen, especially of those organisms which produce organic acids and carbon dioxide, and of mastication, some solution of the three salts is favored. However, since no absorption from the mouth takes place, and since there are ample amounts of fluids in the stomach and in the intestines which favor solution of the inorganic elements, the saliva and the oral cavity do not play an important rôle in the assimilation of the three elements.

According to McGowan, the hydrochloric acid is one of the most important agencies for the absorption of the alkaline earth elements and especially of calcium phosphate. While it cannot be denied that hydrochloric acid may play an important rôle, either directly or indirectly, it is the concentration of hydrogen ions in the upper levels of the small intestines which determines whether the acid or the basic form of calcium phosphate will exist and, since acid calcium phosphate is the more soluble, it is evident that absorption will be favored if this form of calcium is present. In this connection, it should again be pointed out that, since a considerable portion of the ingested phosphorus is in the form of esters and these are but slowly hydrolyzed, calcium will not necessarily be absorbed as calcium phosphate even when calcium and phosphorus are present in the diet in stoichiometric proportions. The gastric acidity of the adult human is about pH 1.0 to 2.5. Under these conditions, compounds of calcium with weak organic acids are converted into the chloride. Calcium carbonate has been recommended for the neutralization of gastric acidity, since any excess beyond that required for neutralization probably in large part escapes absorption and, unlike sodium bicarbonate, will not increase the body's fixed alkali. Some protein digestion takes place in the stomach. This, together with the gastric acidity, serves to convert complex calcium-protein compounds into the inorganic form and effects solution of basic calcium phosphate provided, of course, that the food remains in the stomach for a sufficient length of time to become intimately mixed with the gastric juice. The gastric acidity of the human infant's stomach is about pH 3.5 to 5.0. This acidity is still favorable for the solution of the calcium salts. In the carnivorous animal, particularly the dog, the

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gastric acidity is also high. Very few good data relating to gastric acidity of other animals are available. Presumably, it is sufficiently high to effect solution of the calcium compounds.

While solution of calcium salts by the gastric juice is important, it is by no means an indispensable factor. In hypochlorhydria, the gastric acidity is low and yet there is no serious interference with the assimilation of calcium. It appears possible to neutralize or even alkalinize the gastric juice for some periods of time with such agencies as sodium bicarbonate without leading to any serious interference with the absorption of calcium and probably magnesium as well. Thus Goss and Schmidt found no essential difference in the retention of calcium and phosphorus by rats up to the age of about 70 days irrespective of whether the diet was made alkaline by the addition of sodium bicarbonate or acid by means of hydrochloric acid. It has been found by Mussehl, Hill, Blish, and Ackerson that the growing chick is able to adjust itself to wide ranges of acidity and alkalinity provided an ample supply of vitamin D was furnished. Unpublished experiments in this laboratory by Curry and Schmidt showed that considerable doses of sodium bicarbonate could be administered daily to the rat without interfering with pregnancy and lactation. The effects of an alkaline reaction in the intestines will depend somewhat on the nature of the calcium compounds undergoing absorption. If tertiary calcium phosphate is formed, the amount which is held in solution is very small, and hence the amount which is absorbed will be correspondingly small or negligible. The formation of calcium hydroxide and calcium bicarbonate will not necessarily preclude their absorption since they are somewhat soluble; the rate, however, will be considerably less than if the more soluble forms were present. Absorption will, in large part, depend on the time during which the food remains in the intestines. Givens and Mendel concluded that the addition of acid or base produces no significant effect upon the balance of calcium and phosphorus in dogs, but the addition of hydrochloric acid increases the output of urinary calcium. Similarly, Zucker showed that ingestion of acid led to a shift in both calcium and phosphorus from feces to urine, while the reverse effect was obtained with sodium bicarbonate. The total balance, however, was not changed. The concentration of calcium in gastric juice is about 5 to 7 mgm. per 100 ml.

Except as an agency for effecting solution of calcium phosphate, the gastric juice probably plays no important rôle toward promoting solubility of the esters of phosphoric acid. Ferments which split these

esters are apparently not present in the stomach and, if they were present, they probably would find the reaction of the gastric contents unfavorable. It is generally assumed that the various esters of phosphoric acid must be split in order that absorption can take place, but it by no means proves that this prerequisite is a universal and necessary one, especially in the case of such compounds as serine phosphoric ester, glycerol phosphoric ester, and the various hexose phosphoric esters.

The small intestines, especially the duodenum, are all important for the absorption of calcium salts. The acidity of the duodenum of the carnivorous and omnivorous animal has been reported to vary from pH 2.3 to 7.0; the lower levels of the small intestines vary from pH 5.9 to 6.5. The reaction of the intestines of herbivorous animals is somewhat more alkaline; the range is about pH 6.7 to 8.0. The calcium and magnesium compounds which exist as chlorides, and acid calcium phosphate, are probably absorbed in the upper parts of the small intestines before complete neutralization of the gastric juice has taken place. With the disappearance of free hydrochloric acid due to neutralization, the acidity due to carbon dioxide, as well as the formation of organic acids such as lactic acid by bacterial action, become factors which promote solubility of the calcium salts. All important is the fact that splitting of the various esters of phosphoric acid such as lecithin and the carbohydrate esters does not take place until these substances have been acted upon by the pancreatic juice and the succus entericus. We can infer that a considerable portion of the phosphoric acid is absorbed into the blood stream at a later time than the major portion of the calcium salts. If it were not for this fact, a portion of both the ingested calcium and phosphoric acid would probably not be absorbed, especially in regions of the small intestines where the acidity is low. In this connection, it should be emphasized that both the absorption mechanism and the foods which contain the alkaline earth elements and phosphoric acid constitute heterogeneous systems from the standpoint of chemical reactions. This favors to a considerable extent selective liberation, solution, and absorption of the two alkaline earth elements and of phosphoric acid.

Considerable emphasis has been placed in the literature on the ratio of calcium to phosphorus as one of the factors which affect their metabolism. In rats, as has been shown by McCollum, in addition to a deficiency of vitamin D, it is necessary that the relation of calcium and phosphorus be as 1:6 or 6:1 in order to produce rickets. In most other animals, a deficiency of vitamin D without any upset in Ca/P ratio will

lead to the same condition. Too much emphasis has probably been placed on the ratios of the two elements and not enough consideration has been given to the quantitative aspects of the problem.

Shohl and his co-workers have pointed out that both the level and the ratio of Ca/P are necessary to characterize the ricketogenic properties of a diet. In support of this idea they have shown that at a given level of calcium (or of phosphorus) increasing the ratio of Ca:P intensifies the degree of rickets. When, at any given ratio of Ca:P, the level of these salts is increased, the degree of rickets is diminished. Bethke, Kick, and Wilder found that for optimum growth and bone formation, a Ca:P ratio of 2.0 to 1.0 is best. The vitamin D requirements are then a minimum. The wider the ratio, the greater will be the requirements for vitamin D. Increasing the Ca:P ratio from 1.0 to 5.0 led to a progressive decrease in growth, bone, ash, and percentage of inorganic phosphorus in blood serum. On the other hand, when the Ca/P ratio was changed from 1.0 to 0.25, growth was depressed and the ash content of the femurs was slightly decreased.

It is obvious that many phosphoric acid compounds occur in the soft tissues which are not combined with calcium salts. It is therefore necessary that there be available not only a sufficient amount of phosphorus to supply the needs of these tissues, but also to form the requisite calcium phosphate for skeletal growth. It would seem then that under optimum conditions some of the phosphoric acid should be absorbed in the form of salts other than of calcium. In order to produce metabolic disturbances created by lack of phosphorus, it is necessary that there be ingested a sufficiently large excess of calcium salts for combination with all or most of the phosphoric acid which is contained in the diet. Conditions which favor insolubility of phosphoric acid in the intestinal tract prevent its absorption. Thus, the ingestion of beryllium salts, as has been shown by Kay and his co-workers, leads to a type of rickets caused by the formation of insoluble beryllium phosphate in the intestinal tract, which prevents the absorption of phosphoric acid. Other metallic salts, such as aluminum, thallium, barium, strontium, and magnesium apparently act similarly. It is also very probable that the ingestion of an excess of soluble calcium salts will lead to the removal from the blood stream and the soft tissues of phosphoric acid by forming the less soluble calcium phosphate.

The converse relation, viz., low calcium and high phosphorus, may be a factor in influencing calcium metabolism. Tetany may result if an animal maintained on a diet low in calcium be fed a considerable excess

of phosphoric acid. While an excess of phosphoric acid may contribute to the intestinal acidity, thus presumably aiding the solubility of calcium, it may also draw upon the body's reserve of calcium for purposes of neutralization. The formation of calcium phosphate in the intestinal tract will, in large part, depend on the form in which phosphorus is ingested. The formation of insoluble calcium phosphate may lead to excretion of calcium rather than absorption. The effect of the ingestion of an excess of phosphorus over calcium unless very large will, for short periods of time, not necessarily lead to deleterious effects in the adult animal. In order to maintain the necessary level of diffusible calcium in the blood stream, the body can, in time of need, draw upon the calcium phosphate of which there is a large supply available in the bones. The amount of calcium required to maintain the optimum level in the blood stream is comparatively small. The growing animal is probably influenced to a greater extent than the adult animal by a considerable excess of phosphorus over calcium in the diet due to the fact that the animal needs calcium for skeletal growth. Therefore, the body can less afford a drain of calcium from either the skeleton, soft tissues, or blood stream for purposes of neutralizing phosphoric acid. The effect of an excess of phosphorus in the diet will undoubtedly depend in large part on the particular compound in which phosphorus is combined. Presumably, an alkali salt of phosphorus would have less effect than an acid salt.

According to Fischer and Hendschel, magnesium is split from its combination in chlorophyll in the stomach of the ruminant and thus becomes available for absorption. The magnesium complex of chlorophyll is quite stable in the presence of alkali, but in the presence of acids, magnesium is quantitatively split off and replaced by oxygen. This process takes place to a considerable extent in the presence of the acid of the gastric juice. It is quite probable that the absorption of magnesium follows the same path as that of calcium.

A great many studies, whose object was to elucidate the mechanism and the factors concerned in the absorption of calcium from the intestinal tract, have been carried out. The literature is so large that all of the experimental evidence in favor of the various views which have been advanced cannot be reviewed here. A number of experimentally established facts stand out. Animals which have been maintained for some time on a vitamin D deficient diet are in negative calcium and phosphorus balance. The ingestion of sufficient amounts of vitamin D by such animals changes a negative balance to a positive one. The net

result is to make it appear that vitamin D increases or is prerequisite to the absorption of calcium (and magnesium) and of phosphorus. This is essentially the view which has been expressed by Chick, Harris, and others. The situation, however, is not so simple as might be inferred from the above. To fully confirm this hypothesis, it is necessary to show in what way vitamin D aids in the transport of the elements under consideration. In a rough way and within certain limits, the amount of calcium and of phosphorus which is "absorbed" or, perhaps stated better, "utilized," is proportional to the dose of vitamin D. The required dose of vitamin D has been reported to vary with the calcium and phosphorus ratio. Possibly an adequate hypothesis for vitamin D action on this basis is that it increases the intestinal permeability for the two elements. This statement, however, still does not supply an adequate answer to the question at issue. It has been quite generally established that, despite the administration of sufficiently large doses of vitamin D so that it is present in the milk, heavily lactating animals are usually in negative calcium and phosphorus balance. The answer to this may be that there is a limit to the amount of calcium and phosphorus which can be absorbed, or perhaps that the concentration of vitamin D in this condition is at such a level that calcium and phosphorus are withdrawn from the skeleton rather than deposited therein. However, as will be shown later, there is a possibility that factors other than vitamin D are concerned in calcium absorption. Excessive doses of vitamin D lead to a negative calcium and phosphorus balance. Hypercalcemia may be induced in dogs which are in negative calcium balance or upon calcium-free diets by overdosage of irradiated ergosterol. In this condition there is a considerable increase in the amounts of calcium and of phosphorus which are excreted in the urine. These results would hardly be expected if the action of vitamin D were to increase the permeability of the intestines for the two elements. It is more reasonable to assume that, as a result of overdosage of vitamin D, mobilization of calcium and phosphorus either by the direct action of vitamin D on the skeleton or as a result of the stimulating action of the latter on the parathyroid gland,[5] calcium and phosphorus are

[5] Selye has brought forth evidence which is seemingly at variance with this idea. The effect produced by hyperparathyroidism in young rats differs from that produced under the same condition by hypervitaminosis D. It has not yet been shown that osteitis fibrosa may be induced in animals by vitamin D overdosage. On the other hand, there is a considerable parallelism with respect to blood and urine chemistry and the histological changes in bone, between hyperparathyroidism and osteitis fibrosa. However, further experimental evidence is needed to elucidate the mechanism of vitamin D and parathyroid action.

withdrawn from the bones. A somewhat constant relation between the two elements in the increased amount which is excreted in the urine in hypervitaminosis suggests that these elements are derived from the skeleton. The latter stages of hypervitaminosis D show a suppression in the excretion of calcium via the bowel rather than increase in absorption. No adequate hypothesis has been advanced to explain this phenomenon. While there are no experimental data to support the view, it is quite possible that, as a result of vitamin D overdosage which, in turn, leads to the appearance in the blood stream of colloidal calcium phosphate, deposition of the latter may perhaps take place in the tissues and particularly in the lumen of the large intestines so that excretion via this path is markedly interfered with and reduced. Adequate data would, however, have to demonstrate that under such conditions sufficient deposition of calcium phosphate does not also take place in the kidneys to interfere with the excretion of calcium and phosphorus in the urine.

An hypothesis which, on the basis of the known facts, appears to more adequately explain the action of vitamin D, has been advanced by Taylor and others. The essentials of their view are that vitamin D does not assist in the absorption of calcium and phosphorus from the intestines, but rather in its deposition in the bony framework of the body. A sharp distinction must be made here with respect to the utilization of the two elements. Most of the body's calcium is stored in the bones. While the amount which is present in the blood and in the tissues is vital to normal function, it is nevertheless small. The blood level can be kept within normal limits by mobilization of calcium phosphate from the bones or inflow as a result of intestinal absorption. The regulator which maintains this blood level is probably parathyroid hormone. On the other hand, while a considerable quantity of phosphorus is stored in the bones as calcium phosphate, a large quantity is also present in the soft tissues of the body. It is not primarily associated with calcium in the form of calcium phosphate. From this it follows, on the supposition that calcium and phosphorus are absorbed without the assistance of vitamin D, that a considerable part of this phosphorus, and, to a slight degree, calcium as well, may be utilized by the soft tissues even in the absence of vitamin D. McGowan has suggested that the action of vitamin D consists in setting free inorganic phosphorus endogenously from the lipoids of the body. This theory, while interesting and suggestive, does not explain all of the phenomena which have come to be ascribed to vitamin D. Possibly vitamin D plays two rôles, one in relation to the form of combination of phosphorus in the tissues, and the

other in the deposition of calcium phosphate to form bone. The major portion of calcium and an equivalent amount of phosphorus, even though absorbed, are not retained by the body since, in the absence of vitamin D, deposition or mineralization of osteoid tissue to produce bones does not take place to the same extent as when ample amounts of vitamin D are available. This view finds support from the well established fact that, in the absence of vitamin D, the condition of rickets occurs. Curiously, all animals do not act alike in this respect. As has already been pointed out, in order to induce rickets in the rat, it is necessary not only that the diet be deficient in vitamin D, but also that there be an abnormal relation of calcium to phosphorus. In infants, dogs, and chickens, it appears that rickets may be brought about merely by a deficiency of vitamin D in the diet even though the ratio be a normal one, i.e., equimolecular. The optimum relation of calcium to phosphorus for the pregnant and lactating rat, as has been recently pointed out by Cox and Imboden, is 1:1 or 1.5:1.

No adequate explanation has been advanced to account for the natural resistance of the rat to rickets. The greater need for phosphorus by the chicken may perhaps be ascribed to the greater demand of the soft tissues for this element rather than a difference in the relations of calcium and phosphorus in the bones. In this connection it is of interest to mention that recent evidence suggests that natural vitamin obtained from fish oils and the antirachitic substance obtained by irradiating ergosterol are probably chemically and physiologically different.

In certain of the experiments reported by Taylor, Weld, and Sykes, it was found that, irrespective of whether dogs were starved or given moderate doses of calcium chloride, the calcium content of the feces remained nearly constant. In subsequent experiments, it was found that, on further increasing the dose of calcium to about 3.5 grams, while an increase in fecal excretion occurred, it was not proportional to the increase in the input and apparently was not affected by moderate doses of irradiated ergosterol. On continued administration of calcium, the fecal excretion was equal to or slightly greater than the input of calcium. Very little difference in urinary calcium excretion was noted until the amount of ingested calcium was a gram or more. The average daily urinary calcium did not exceed 350 mgm. No experiments were carried out on rachitic animals. Several workers, among them Harris, and Bills and Wirick, have reported that a high calcium diet increases the hypercalcemia and the toxic effects of irradiated ergosterol. The

calcifying action of vitamin D is enhanced by a diet high in calcium. These facts do not necessarily indicate that the function of vitamin D is to increase calcium absorption. It seems quite plausible that if, as the result of an overdosage of vitamin D, calcium is withdrawn from the skeleton and, due to the high calcium input, the inflow of calcium is somewhat greater than normal, the sum total effect of these two processes is to decidedly raise the level of serum calcium. On a non-toxic dose of vitamin D it is to be expected that when the calcium inflow is raised as a result of increasing the calcium of the diet, calcification will take place at a more nearly optimum than if the inflow were maintained at a lower level.

According to Shelling and Asher, optimal calcium-high phosphorus diets are the most predisposing to the injurious effects of viosterol, while high calcium-low phosphorus and very low calcium-optimal phosphorus diets are the least effective. Vitamin D is markedly effective in protecting rats from losses of calcium when they are maintained on diets which are low in this element. There is, of course, a limit in the capacity of the vitamin in affording this protection.

It is doubtful whether any hypothesis which has so far been advanced to explain the mechanism of calcium absorption and the relation of vitamin D thereon is adequate. Experimentally, it is difficult to segregate the various factors concerned. The intestines are the chief organ for absorption as well as for excretion of calcium. The amount of calcium which appears in the feces is the sum of that which is excreted and that which is unabsorbed. It is not easy to determine whether all of that which appears in the feces represents in addition to the endogenous output unabsorbed calcium or calcium which has been absorbed and not utilized. The negative balances in lactating animals suggest that there is a limit in their capacity to absorb calcium, or, if it be absorbed, it cannot be utilized for purposes of supplying this element to the milk. The relation of vitamin D to calcium absorption is also not easily solved since this substance apparently possesses several functions. Primarily, it is concerned in bone formation. However, a large dosage will remove calcium from the skeleton. We are faced with the dilemma in attempting to ascertain whether or not vitamin D assists calcium absorption, in segregating its effect in promoting calcification, its possible effect in stimulating secretion of parathyroid hormone, its decalcifying action when the dosage is increased and its possible influence in promoting absorption.

It is the belief of the writers that sufficient attention has not been

directed to the endogenous calcium and phosphorus metabolism of the skeleton. Aub and his associates have indicated that this is a factor of considerable importance. In inanition and in calcium-free diets, a considerable amount of calcium and phosphorus is excreted. The reservoir which contains these elements is large. The amount of calcium excreted by animals which have been maintained for some time on calcium free diets stands in contrast to that of the alkali elements and chlorine. The latter elements, if withheld from the diet, practically disappear from the excreta. On the other hand, the amount of calcium and phosphorus which is excreted remains at a high level despite a low input level. The endogenous metabolism of calcium and phosphorus may in many ways be compared with that of nitrogen. The amounts of both excretory products are considerable. It seems quite reasonable to assume that in a full grown animal there is a limit to the amount of calcium which the body needs and can store. Under equilibrium conditions this is also true of nitrogen. The reservoirs for both elements are large and, in time of need, as in inanition and in lactation, etc., they can continue for long periods of time to give up their stores. There are, however, two essential differences between calcium and nitrogen metabolism. For deposition of calcium, vitamin D must be supplied in the diet. An overdosage leads to withdrawal of calcium from the skeleton. No known dietary factor appears to be directly essential for protein synthesis and storage. Fortunately, for purposes of estimating the amount of protein which is utilized and in determining the endogenous and exogenous fractions, the nitrogen is excreted chiefly by way of the urine, although for accurate purposes, cutaneous elimination, etc., which, however, is small, must be considered. On the other hand, the chief avenue for calcium and phosphorus excretion is the same as that for absorption. The fecal output represents then both the metabolized elements and the non-absorbed fraction of the diet. This makes a differentiation, such as is easily possible in the case of nitrogen, very difficult if not impossible.

There are experimental indications that there may be a dietary factor which, however, is apparently not vitamin D, which assists in the transport of calcium and possibly also phosphorus across the intestinal wall. Much of this work has been carried out by Hart, Steenbock, and their associates.

It has been quite definitely established that high lactating animals are in negative calcium and usually also phosphorus balance. Thus, Forbes and his co-workers found that in 49 balance trials during liberal

milk production without exception all of the animals were in negative calcium, phosphorus, and magnesium balances. Supplementing the diet with calcium salts did not alter the negative balances, indicating that the animal can absorb only a limited amount of calcium, or, if the calcium is absorbed, it is not utilized. The calcium balances changed from negative to positive during the period of pregnancy when the cows were dry. The phosphorus balances were in most cases also negative, but not invariably so, indicating that the conditions for the assimilation of this element may be more readily attained than in the case of calcium. The experiments of Goss and Schmidt on rats are probably typical of the metabolism of calcium and phosphorus in most animals during pregnancy and lactation. They showed that the majority of their animals stored both calcium and phosphorus during the period of pregnancy. During lactation the input of both calcium and phosphorus decidedly increased, but the increase was not sufficient to balance that which was eliminated in the milk and the animals were therefore in negative calcium and phosphorus balance. Within certain ranges, acidity did not influence calcium and phosphorus assimilation. It was evident that those elements were in all probability withdrawn from the skeleton. Goss has since been able to show that he could withdraw about 60 to 70 per cent of the total calcium contained in the body of the rat by placing the animal on a low calcium diet and subjecting it repeatedly to pregnancy and lactation. He has been further able to show that these depleted rats both during the rest period and pregnancy can store about a milligram of calcium per day when the input was as low as 7 or 8 milligrams, but invariably during lactation negative balances resulted. The data of Brooke and Smith are perhaps not so striking as these, but they also indicate that rats can be kept at fairly low calcium levels with comparatively little loss.

It has been the object of the Wisconsin group of workers to ascertain whether it is possible by dietary measures to maintain animals in calcium equilibrium during the period of lactation. They found in general that the addition of calcium salts led to some improvement in the balances of cows maintained on a dry ration with the addition of corn silage, but equilibrium was not secured. Likewise, addition of considerable amounts of vitamin D in the form of cod liver oil, irradiated yeast, or exposure of the animals to ultra violet light did not place heavily lactating cows in calcium equilibrium. Vitamin C in the form of orange juice or cabbage was also without material influence. The addition of HCl to the diet led to an increase in the urinary calcium

excretion, but did not favorably influence the calcium balances. Glucose was without effect. On the other hand, they found that cows yielding about 50 pounds of milk per day could be kept in positive calcium balance when the ration consisted of about 40 pounds of green grass, 30 pounds of corn silage, and a grain mixture of 59 parts of yellow corn, 25 of wheat bran, 15 of oil meal, with liberal addition of a calcium salt. Fresh alfalfa was particularly effective. From their experiments they conclude that it is altogether possible that there are factors other than antirachitic vitamin operating in the absorption of calcium from the intestinal tract of the cow. There are not as yet sufficient data at hand to indicate the nature of such a possible factor or its properties. Experiments of this type are of very great practical importance since, if a factor could be found which would materially assist in the assimilation of calcium and phosphorus, it would be possible to maintain heavy producing cows without the danger of death resulting from the strain of excessive drainage of mineral and other constituents of the body. The tendency of breeding has been to develop animals largely for heavy milk production without regard to the possibility of bringing out by similar procedures anatomical factors which might increase the assimilation of calcium and phosphorus. Balance studies of lactating animals illustrate a general biological axiom that the mother will sacrifice her own tissues to supply, if possible, the needs of her offspring.

An interesting series of experiments was recently reported by Greaves and Schmidt. They are illustrative of the many factors which must at times be considered before a clear picture of physiological processes can be obtained. They showed that bile is necessary for vitamin D absorption when the latter is ingested by mouth. Rachitic rats with internal fistulas so that little or no bile was present in the small intestines were in negative calcium and phosphorus balance despite the oral administration of irradiated ergosterol. The administration of deoxycholic acid along with the irradiated ergosterol restored the animals to a positive balance. On the other hand, rachitic rats with internal bile fistulas could be induced to store calcium and phosphorus by administering the irradiated ergosterol subcutaneously. Taylor's experiments point in the same direction. He obtained no blood hypercalcemia when vitamin D was fed to bile fistula dogs although injection did produce it. Bile collected from bile fistula dogs exhibited antirachitic properties when vitamin D was administered intraparenterally, but not when given by mouth. The bile itself does not constitute a channel for the excretion of any considerable amounts of calcium,

magnesium, or phosphorus. According to Ravdin, Johnston, Riegel and Wright, the calcium content of hepatic bile varies between 5.3 and 9.7, while the range for gall-bladder bile is 6.9 to 25.7 milliequivalents per liter. These figures are in harmony with the generally accepted idea that the calcium content of bile may be altered by the gall bladder, by concentration. The calcium and magnesium content of bile is not markedly influenced by dietary intake; it is more nearly related to the blood level of these elements. According to a number of Japanese workers, cholic acid increases calcium absorption. There is an increased level of blood calcium, magnesium, and an increased urinary excretion of calcium. This effect, however, may be an indirect one, possibly dependent on the absorption of vitamin D.

2. FATE IN THE BLOOD STREAM. After their absorption from the intestines, the alkali earth elements find their way into the blood stream and other fluids of the body. The exact route from the intestines to the general circulation is not known. Speculatively, one may assume that the portal system is the important route by which these substances reach the general circulation. Another route may be through the lymphatic system and via the thoracic duct into the blood. What part this path plays, if any, in the transport appears to be completely unknown. Necessarily, the exchange of these elements among the different tissues of the body takes place by means of the blood and the other body fluids. It is important therefore to survey the amount, the state, and the factors which influence the level of the calcium, the magnesium, and the phosphorus compounds of the blood and the other body fluids.

a. The state of blood calcium. The blood will be considered first. This has undoubtedly been the most extensively and intensively studied material. All of the complexities of the subject, so far as they are now known, are represented in the blood to the highest degree. In the first instance, since the blood is not a macro-homogeneous fluid, it has to be considered with regard to its two components, the corpuscles and the plasma. Since there is apparently no interchange of cations between plasma and corpuscles, excepting for hydrogen ions, the two can be considered separately.

With calcium a still further simplification is attained, because the most reliable work points to the presence of either only a mere trace or no calcium in the corpuscles, the total amount of this element being confined to the plasma. This observation was first made on the blood of a large number of mammals by Abderhalden in 1898. It has since been substantiated by many workers, among whom may be mentioned

Rona and Takahashi, Kramer and Tisdall, and most recently, Rymer and Lewis, Gulácsy and Schönberger. Rymer and Lewis found on the average about 0.5 mgm. of calcium per 100 ml. of blood to be present in the corpuscles of both normal subjects and psychopathic patients. The work of Gulácsy and of Schönberger was especially carefully carried out, the calcium being determined on whole blood and plasma which was evaporated with HCl and then carefully ashed before analysis. The data offered by these authors show results of the same order as found by Rymer and Lewis, but these small figures, they feel, are actually due to experimental shortcomings. In the investigations quoted, as in practically all of the investigations of the subject, the corpuscles were not directly analyzed for calcium, but instead, the amount was calculated from the whole blood and the plasma values, based on the fraction of the blood volume occupied by the corpuscles. Stewart and Percival report some 0.2 to 0.3 mgm. per cent of calcium by direct analysis of corpuscles washed free from plasma with saline solution. Thomas adds a discordant note to the almost universal acceptance of the idea that there is no calcium present in the erythrocytes of mammals. According to this author, while there is practically no calcium present in the corpuscles of man and of the pig, the cells of the ox and of the sheep contain from 0.6 to 5.5 mgm. per cent.

Bär has suggested that, while ordinarily there is practically no calcium present in human corpuscles in certain pathological conditions, particularly in tetany, a migration of calcium into the red cells takes place. Morgulis and Perley found no increase in the corpuscle calcium of parathyroidectomized dogs. They also point out that there is no basis for the claim of Bär as shown by his own data. The trend of the evidence is such that very strong proof will have to be offered to counteract the feeling that there is no more than a trace of calcium present in the corpuscles of mammals.

In actual practice, analysis of blood calcium is almost invariably carried out on blood serum. The reason for this is fairly self evident. In order to obtain stable plasma, the addition of some foreign anticoagulant is required which is more often than not objectionable. However, this raises the question as to whether serum gives an adequate representation of the state of affairs in the living animal, namely, is there any significant difference between the calcium content of the serum and of the plasma of the same blood? Plasma obtained from citrated blood contains practically the same amount as or a little less calcium than is found in the serum. Citrates, it is well known, produce

some shrinking of the corpuscles, which leads to a small dilution of the plasma. Stewart and Percival believe that the agreement between this type of plasma and the content of serum results because of the precipitation of an unknown form of calcium by the added citrates. In true plasma, obtained without anti-coagulants, there is present, according to these authors, from 1 to 2 mg. per cent more of calcium than in the corresponding serum. Öwre similarly finds about 1 mgm. per cent more calcium in plasma.

Since the bulk of the analytical work has been carried out on the serum of the blood, our main attention needs to be given to the results obtained with this menstruum. The serum calcium has been determined on many thousand samples of blood from man and other animals. Numerous figures have been published on the blood calcium of both normal animals and those suffering from almost every conceivable pathological condition. The astonishing fact is the great constancy that has been obtained for the serum calcium values in health and also in most diseased states. A summary of the distribution of calcium along with related substances in the blood of a number of animal species is given in tables 5, 6 and 7. It seems highly probable, as shown in table 5, that the range of variation for healthy human subjects lies between 9 and 11.5 mgm. per cent. Most series determinations on small groups, carried out in order to fix a normal value as a guiding basis, show less variation than the above. Such series, usually selected from among laboratory associates of closely the same age, are apt to be misleading. The analyses reported by different investigators may show greater variations than have been reported by one single analyst. Some of this difference is undoubtedly due to differences in the analytical methods employed.

The most widely used method of analysis in the English speaking countries is the one developed by Kramer and Tisdall in which calcium is directly precipitated from the serum. Minor variations have been introduced by many persons. According to Van Slyke and Sendroy, from 5 to 15 per cent more calcium is obtained by the analysis of trichloroacetic acid filtrates of serum than of serum directly. The micro filtration procedure, introduced by Kirk and Schmidt, eliminates the difficulties of washing the precipitate by centrifugation. This is especially true when protein-free fluids, such as serum ultrafiltrates or spinal fluids, are to be analyzed. In these fluids, films of finely divided calcium oxalate persist in floating on the surface, even after prolonged and rapid centrifugation, with a consequent analytical loss. Fisk and

Logan have developed a method based on igniting the calcium oxalate to calcium carbonate and oxide and titrating alkalimetrically.

Further consideration of the blood calcium level of different animal species and of factors influencing the level and distribution will be deferred until the physical chemical state of the blood calcium is surveyed.

TABLE 5

The distribution of the alkali earth elements and of interrelated substances in the blood of human subjects

SUBSTANCE	NUMBER OF OBSERVATIONS	RANGE*	NORMAL MEAN	STANDARD DEVIATION	SOURCE OF DATA
Total serum calcium	42	9.0–11.4	10.3	0.5	(1)
Diffusible calcium	42	4.4– 6.8	5.4	0.5	(1)
Diffusible calcium, per cent of total calcium	42	40–64	53	5.0	(1)
Non-diffusible calcium	39	3.4– 5.8	4.9	0.6	(1)
Total serum protein, per cent	29	6.0– 8.4	7.2	0.6	(1)
Albumin, per cent	28	4.0– 6.0	5.0	0.5	(1)
Globulin, per cent	23	1.4– 3.0	2.2	0.1	(1)
Inorganic serum phosphate	33	3.0– 5.8	4.0	0.6	(1)
Whole blood magnesium	52	3.4– 5.4	4.6	0.4	(2)
Red corpuscle magnesium	58	5.4– 7.8	6.6	0.5	(2)
Plasma magnesium	58	2.0– 3.6	2.7	0.3	(2)
Serum magnesium	41	1.8– 3.2	2.5	0.3	(3)
Diffusible serum magnesium	41	1.4– 2.4	1.9	0.2	(3)
Diffusible serum magnesium per cent	41	60–90	80	7.0	(3)

* Figures for inorganic substances are expressed in milligrams of element per 100 ml.

(1) Combined values of E. WATCHORN AND R. A. MCCANCE. Biochem. J. **26:** 54, 1932; J. G. WOOLEY AND H. ROSS. U. S. Pub. Health Repts. **47:** 380, 1932; and L. GUNTHER AND D. M. GREENBERG. Arch. Int. Med. **45:** 983, 1930.

(2) GREENBERG, D. M., S. P. LUCIA, M. A. MACKEY AND E. V. TUFTS. J. Biol. Chem. **100:** 139, 1933.

(3) WATCHORN, E. AND R. A. MCCANCE. Biochem. J. **26:** 54, 1932.

b. Physical chemical state. For a complete characterization, it is not sufficient to consider the serum calcium as if it exists in a unitary condition and so merely treat it in toto. In 1911, Rona and Takahashi demonstrated, by dialyzing serum against saline solutions containing variable amounts of calcium, that only a portion of the calcium was

dialyzable. Since then a large amount of work has appeared on the forms of calcium in biological fluids. With no desire to detract from the

TABLE 6

The distribution of the alkali earth elements and of interrelated substances in the blood, and a comparison of their distribution in the sera of normal dogs as obtained by ultrafiltration and by vividialysis

SUBSTANCE	NUMBER OF OBSERVATIONS	RANGE*	MEAN	STANDARD DEVIATION	SOURCE OF DATA
(a) By ultrafiltration					
Serum calcium	31	9.0–13.0	10.8	0.6	(1)
Diffusible calcium	31	4.5– 8.5	6.5	0.7	(1)
Diffusible calcium, per cent of total calcium	31	50– 75	61.5	5.5	(1)
Non diffusible calcium	31	2.0– 7.0	4.4	0.9	(1)
Inorganic phosphate	27	3.0– 8.5	4.9	1.0	(1)
Diffusible inorganic phosphate	24	3.0– 9.0	4.8	0.9	(1)
Total plasma proteins,† per cent.	50	6.4– 8.2	7.0	0.4	(2)
Albumin, per cent	50	4.0– 5.6	4.4	0.3	(2)
Globulin, per cent	50	1.8– 2.9	2.1	0.2	(2)
Plasma or serum magnesium	43	1.6– 3.4	2.3	0.2	(1)
Diffusible serum magnesium	9	1.0– 2.0	1.5	0.4	(1)
Red corpuscle magnesium	22	7.0–10.5	8.1	0.9	(1)
(b) By vividialysis					
Serum calcium	14	8.5–12.5	10.5	1.0	(3)
Diffusible calcium	14	5.0– 8.0	6.3	0.6	(3)
Diffusible calcium, per cent	14	50– 75	62	6.0	(3)
Non diffusible calcium	14	2.0– 6.0	4.2	1.3	(3)
Inorganic phosphate	14	2.0– 8.0	5.0	1.5	(3)
Diffusible inorganic phosphate	13	2.0– 7.0	4.8	1.2	(3)
Total serum protein, per cent	15	5.5– 8.5	6.6	0.7	(3)
Serum magnesium	12	1.0– 3.0	2.2	0.5	(3)
Diffusible magnesium	12	0.5– 2.5	1.7	0.7	(3)

* Figures for inorganic substances are expressed in milligrams of element per 100 ml.

† Includes fibrinogen of the plasma.

(1) GREENBERG, D. M. AND CO-WORKERS. Unpublished data.
(2) MATHEWS, C. W. J. Biol. Chem. **74:** 557, 1927.
(3) GREENE, C. H. AND M. H. POWERS. J. Biol. Chem. **91:** 183, 1931.

sound and valuable work in this field, there has been a marked tendency to postulate the existence of special forms of calcium having specific physiological properties on the most tenuous grounds. On the other

TABLE 7

The distribution of the alkali earth elements and of interrelated substances in the blood of certain animals

SUBSTANCE	NUMBER OF OBSERVATIONS	RANGE*	MEAN	STANDARD DEVIATION	SOURCE OF DATA
Rabbits					
Total serum calcium	33	11.0–16.0	13.1	1.2	(1)
Diffusible calcium	33	7.0–10.0	8.5	0.8	(1)
Diffusible calcium, per cent of total calcium	33	45– 80	67	6.0	(1)
Non-diffusible calcium	28	3.0– 6.0	4.2	0.8	(1)
Serum calcium	80	14.5–18.5	15.5		(2)
Serum magnesium	49	1.5– 4.0	2.5	0.6	(3)
Inorganic serum phosphate	80	5.0– 6.8	5.8	1.1	(2)
Rat (immature)					
Total serum calcium	60	10.8–12.3	11.3	0.5	(4)
Diffusible calcium	60	5.8– 7.4	6.4	0.5	(4)
Inorganic serum phosphate	60	9.4–12.2	10.3	1.0	(4)
Diffusible serum phosphate	60	9.4–11.6	9.6	0.9	(4)
Guinea pig					
Total serum calcium	9	8.4–10.4	9.4		(5)
Diffusible calcium	9	4.6– 6.6	5.3		(5)
Diffusible calcium, per cent of total calcium	9	52– 60	55		(5)
Hen (non-laying)					
Total serum calcium	5	11.8–13.0	12.2		(6)
Diffusible calcium	5	7.1– 9.3	8.0		(6)
Diffusible calcium, per cent of total calcium	5	55– 77	65		(6)
Inorganic serum phosphate	5	2.0– 4.6	2.9		(6)
Diffusible serum phosphate	5	2.0– 3.7	2.8		(6)
Serum magnesium:					
(hen)			3.4		(7)
(rooster)			2.9		(7)
Hen (laying)					
Serum calcium	7	16.3–25.8	20.7		(6)
Diffusible calcium	7	7.4– 8.6	8.0		(6)
Diffusible calcium, per cent of total calcium	7	29– 46	39		(6)
Inorganic serum phosphate	7	2.3– 4.9	4.1		(6)
Diffusible serum phosphate	7	1.8– 3.9	2.2		(6)

TABLE 7—*Concluded*

SUBSTANCE	NUMBER OF OBSERVATIONS	RANGE*	MEAN	STANDARD DEVIATION	SOURCE OF DATA
		Beef			
Serum calcium	180		12.9	1.7	(8)
Serum calcium		8.8–10.0	9.4		(9)
Diffusible calcium	15		4.9	0.9	(9)
Inorganic serum phosphate	180		4.9	2.7	(8)
Serum magnesium		1.65–2.10	1.7		(9)

* Figures for inorganic substances are expressed in milligrams of element per 100 ml.

(1) UPDEGRAFF, H., D. M. GREENBERG AND C. W. CLARK. J. Biol. Chem. **71**: 87, 1926.
(2) HARNES, A. R. J. Exp. Med. **48**: 549, 1928; **49**: 287, 1929.
(3) BROOKFIELD, R. W. Biochem. J. **27**: 173, 1933.
(4) BENJAMIN, H. R. AND A. F. HESS. J. Biol. Chem. **100**: 27, 1933.
(5) BROWN, H. AND S. G. RAMSDELL. J. Exp. Med. **49**: 705, 1929.
(6) LASKOWSKI, M. Biochem. Ztschr. **260**: 230, 1933.
(7) CHARLES, E. AND L. HOGBEN. Quart. J. Exp. Physiol. **23**: 343, 1933.
(8) PALMER, L. S., R. A. GORTNER AND R. RUDE. J. Dairy Sci., **13**: 360, 1930.
(9) SJOLLEMA, B. AND L. SEEKLES. Biochem. Ztschr. **229**: 358, 1930.

hand, there is a marked tendency in other quarters to lightly regard or totally ignore the possible importance of the existence of more than one form of calcium in blood serum. The endeavor here will be to reach a just and conservative estimate of the value of present day knowledge on this topic.

That there are at least two types of serum calcium components, commonly termed diffusible and non-diffusible calcium, is certain. These are distinguished by the ability of the diffusible fraction to pass readily through membranes impermeable to colloids, while the non-diffusible calcium is held back by such membranes. To determine the quantitative amounts of these two forms of calcium, two kinds of procedures are generally employed, namely, compensation dialysis and ultrafiltration. In compensation dialysis, the procedure is to dialyze serum against a saline solution equivalent in kind and in amount to the salts contained in the blood excepting for the specific ion under investigation. This—e.g., calcium—is varied in order to obtain the level at which there is no further alteration produced by the exchange between serum and salt solution. The non-diffusible calcium does not exist as a firmly bound molecular carbon compound, but, on the contrary, is pres-

ent in an easily dissociable form apparently capable of coming into fairly ready equilibrium with the diffusible calcium. In order, then, to determine the amounts of diffusible and non-diffusible calcium as they exist in the blood, the method employed must be one that does not alter the balance. It is readily understood that when a diffusion equilibrium is established between serum and the dialyzing fluid, there is set up a Donnan membrane distribution of the electrolytes of the serum.

The alternative method of differentiating between these two calcium components is by ultrafiltration. In this method, a portion of the serum is filtered through a collodion membrane by applying a sufficient hydrostatic pressure, and the colloid free fluid so obtained is then analyzed. As stated by Greenberg and Gunther, this procedure was first used as an empirical method which, for no known theoretical reasons, gave similar results to those obtained by compensation dialysis or by analysis of colloid free body fluids. Since then, Greenberg and Greenberg have demonstrated that the ultrafiltration of electrolytes from solutions containing electrically charged colloids leads to a distribution of electrolytes between colloid and ultrafiltrate analogous to that predicted from a Donnan membrane equilibrium. This finding has been substantiated by McBain and McClatchie and also by Ingraham, Lombard, and Visscher. Some difference of opinion exists as to the basic cause of this phenomenon in ultrafiltration. D. M. and M. M. Greenberg and McBain and McClatchie hold to the opinion that this is a consequence of the actual distribution of electrolytes between the colloidal micelles and intermicellar fluid, while Ingraham, Lombard, and Visscher feel that a diffusion equilibrium between colloidal solution and ultrafiltrate during the ultrafiltration is responsible. If the two procedures are analogous in principle, they should yield similar results. This, up to recently, has seemingly not been the case. The most commonly quoted objection to the value of the calcium partition is the supposed difference in magnitude so obtained.

It is generally reported from compensation dialysis analyses that some 60 to 70 per cent of the serum calcium is diffusible. Von Meysenburg, Pappenheimer, Zucker and Murray have reported figures of this magnitude for normal human blood. However, by ultrafiltration, as is seen from table 5, the diffusible calcium is more commonly determined as ranging from 40 to 60 per cent. This discrepancy is not so serious for the claim that the two procedures are analogous in principle as would appear at first sight. Many reasons might be adduced to show that compensation dialysis, as ordinarily carried out in vitro, is open to a

good deal of criticism. The difficulty of preparing an exactly balanced dialyzing fluid, and the fact that no counter pressure is employed to offset the colloidal osmotic pressure of the serum are the most important objections. Rona and his co-workers merely used a sodium chloride solution with varying amounts of calcium as dialyzing fluid. The ultrafiltration procedure is not open to these criticisms.

The highest perfection of the compensation dialysis method has been attained by the use of vivi-diffusion. Here with the total blood of the body as a reservoir for dialysis, the previous objections fall out and the highest accuracy may be expected. A comparison of the results obtained by vivi-dialysis with those by ultrafiltration offers a good test of the agreement expected from the theory. A very careful series of vivi-dialysis experiments on 15 dogs has been carried out by Greene and Powers. For obvious reasons, no similar figures are available on human subjects. Table 6 gives a comparison of the data of Greene and Powers and the results obtained in our laboratory. From the figures presented it is seen that the average agreement by the two methods is astonishingly good. The mean values found by Greene and Powers were: total calcium, 10.5 $\pm$1.0, diffusible calcium, 6.3 $\pm$0.6 mgm. per cent. The figures obtained by one of us gave: total calcium, 10.8 $\pm$0.6, and diffusible calcium, 6.5 $\pm$0.7 mgm. per cent. The average percentage of diffusible calcium is practically 62 per cent in both series. This comparison offers a good check of the essential agreement between the two types of procedure. The older in-vitro compensation dialysis determinations are probably to be regarded as having only semiquantitative significance.

A belief has long persisted and cropped up in various publications, (see Stewart and Percival), that variation in the pressure employed in ultrafiltration might in some way affect—through the alleged decomposition of a labile compound—the value obtained for the calcium distribution in serum. Thermodynamically, for moderate pressures, there is nothing to recommend this idea. The influence of pressure on the equilibrium constant is a function of the compressibility of the components of the solution. With the slight compressibility of aqueous solutions, measurable changes in equilibrium should require enormous pressure differences. Experimentally, the problem has been approached by Nicholas. He employed no. 300 cellophane for the membrane, and found that the calcium in the ultrafiltrate remained quite constant when the pressure was altered from 50 pounds to 200 pounds per square inch. The diffusible calcium on human subjects found with this appa-

ratus, though, was considerably higher—average 64 per cent—than is obtained by the low pressure procedure through collodion membranes.

The pH of the serum and its resulting effect on the relation between diffusible and non-diffusible calcium has also been a subject of considerable comment. The work of at least three investigators indicates that there is only a slight change produced by pH shifts between 7 and 8. In a recent paper, however, Dillman and Visscher claim that pH changes of this magnitude do produce a marked alteration in the distribution of calcium.

It is now necessary to examine the state of the calcium in each of the fractions.

c. Nature of non-diffusible calcium. Since the work of Rona and Takahashi, it has been widely held that the non-diffusible calcium exists in combination with the plasma proteins. A great deal of evidence exists for this belief, although, as will be shown later, at least one form of non-diffusible calcium other than that which is protein bound may, under certain conditions, become important.

An examination of body fluids shows that the calcium content becomes elevated with increasing protein content of the fluid. Thus cerebrospinal fluid, with practically no protein, has the lowest value, while lymph, with about one half the content of protein which is present in plasma, is intermediate between spinal fluid and blood serum. The calcium content of transudates and exudates increases with the amount of the protein. The calcium content of blood which is low in serum protein, as in nephrosis, has long been known to be lower than normal.

Experimental work on the serum proteins in this connection has been carried out by Loeb, Loeb and Nichols, and Marrack and Thacker. The ability of the calcium-protein combination to dissociate readily was demonstrated by its complete diffusion from serum when dialyzed against a very large volume of physiological NaCl solution at a pH of 7.4. The ratio of calcium in the protein solution to calcium in the dialyzing fluid, it was found, is a function of both the protein content and the pH. On the alkaline side of the isoelectric point, the calcium concentration is greater in the protein solution than in the dialyzing fluid. This relationship is reversed on the acid side. On the alkaline side of the isoelectric point, the calcium ratio increases with increasing protein content.

These relations were demonstrated for purified serum albumin and globulin as well as whole serum and also hold true for crystallized egg albumin. The experiments support the idea that calcium forms non-diffusible compounds with the proteins present in blood serum.

The existence of forms of non-diffusible calcium compounds other than those formed with the proteins has also been suggested. Watchorn and McCance have proposed the phospho-lipids as a possible contributing factor. Certain evidence indicating the negligible existence of lipoidal-containing calcium compounds is offered by Loeb and Nichols from a comparison of the calcium binding of untreated and ether extracted serum. Although ether-extracted serum did show a little less binding, the difference between the two was small.

The combination of calcium with the proteins in the non-diffusible fraction is to the greater extent not the usual ionized relationship found with the alkali proteinates. From consideration of the Donnan membrane distribution, it is true that the ionic level of the calcium in the serum should be higher than is found in the colloid-free dialysate or serum filtrate. However, it has been pointed out by Loeb and Marrack and Thacker that the level of the calcium in the serum in a dialysis experiment is too great to be accounted for merely by a Donnan distribution. A similar relationship has been pointed out in ultrafiltration experiments by Greenberg and Gunther.

That the calcium is not in the usual ionized state is also supported by electrical migration experiments in which it has been shown that there is a migration of some of the calcium toward the anode in solutions of the serum proteins. From this reasoning, the logical conclusion is that the calcium in the non-diffusible fraction is combined with the proteins as part of a negatively charged ion schematically represented by the formula,

$$B_nPCa_m = NB^+ + PCa_m^{-n}$$

in which B represents the monovalent bases and P the proteins of the serum. A precedent for this kind of an ion is given by the work of Greenberg and Schmidt and Miyamoto and Schmidt on the alkali earth caseinates. On the basis of conductivity and transference experiments, the existence of similar complex ions was demonstrated in these researches.

Evidence points to the fact that the serum albumin rather than the serum globulin contributes most largely to the binding of calcium. In pathological conditions where a decrease in the concentration of calcium is due to hypoproteinemia, the decrease parallels the fall in the serum albumin. This is true in nephrosis where the calcium falls as the albumin is reduced, even though the globulin may be considerably increased.

A similar parallelism between the albumin content has been noted in

jaundice and neoplastic disease by Gunther and Greenberg. Another line of evidence for this is the following. Upon fractionating the serum proteins, Csapo and Faubl found that more calcium is carried down with the albumin fraction than with the globulin. Bendien and Snapper, by an ultrafiltration procedure with graded membranes which permit the filtration of albumin but of only small amounts of globulin, came to the conclusion that the non-diffusible calcium is completely united to the albumin and none at all with globulin. This seems surprising as one would expect a combination with the two proteins in proportion to the number of acid groups in each.

Under certain conditions, a form of non-diffusible calcium may exist in the blood which is not directly connected with the serum proteins. This apparently is a colloidal form of calcium phosphate. Its existence experimentally is best demonstrated by a concomitant decrease in the diffusibility of both serum calcium and serum phosphate.

The suggested presence of such a substance in blood dates from work by Eichholtz and Starling in 1925 on the influence of calcium salts on the secretion of inorganic phosphate by the isolated kidney. Since then, the formation and nature of this colloidal material has been studied by Grollman, Brull, Scholtz, Laskowski, Smith, and Greenberg and Tufts. The colloidal calcium phosphate is formed both "in vivo" and "in vitro," when blood is considerably augmented with either calcium or phosphate ions. In drawn blood serum, as the calcium is augmented, Greenberg and Tufts (unpublished work) observed a decrease in the diffusibility of the inorganic phosphate parallel with the increase in non-diffusible calcium. After a level of calcium is reached at which the phosphate has become completely non-diffusible, there occurs no further increase in non-diffusible calcium.

Following the intravenous injection of a soluble calcium salt, the change in non-diffusible phosphate, which initially is usually zero, is found to parallel both the height of the serum calcium and the increase in non-diffusible calcium. The colloidal substance commonly appears to have the approximate composition of tertiary calcium phosphate. In the living animal, the formation of this colloid is induced when the calcium is largely augmented by the injection of a soluble calcium salt, by parathyroid extract injection, or by overdosage with irradiated ergosterol. It is also formed when the calcium is normal and the phosphate is highly augmented as by phosphate injections.

However, it would appear that certain levels of the amount of calcium and phosphate in blood have to be exceeded before colloidal calcium

phosphate is formed. With increases of only 2 to 3 mgm. per cent in the blood calcium, induced either by calcium salt ingestion or injection of parathyroid extract, the phosphate retains its complete diffusibility and the rise in blood calcium is reflected almost in toto in the diffusible fraction.

The factors governing the formation of colloidal calcium phosphate can only be surmised for the present, largely because of the lack of exact knowledge regarding the state and the solubility of the bone salts. It seems quite likely, however, that the substance is formed as a stage on the road to the precipitation of a calcium and phosphate containing salt, and it is kept dispersed through the stabilizing and protective action of the serum proteins.

The prevention of the immediate formation of a macroscopic precipitate by the formation of this colloid through the protective action of the serum proteins may be of note in protecting the organism against temporary increases in the calcium phosphate level. However, since the colloid appears to be essentially unstable, if the augmented calcium phosphate level persists for a long time, a slow macroscopic precipitation may take place. It is intriguing in this respect that the two agencies which are particularly associated with experimental metastatic calcification, namely, high dosage of parathyroid extract and irradiated ergosterol, both induce the formation and persistence of the calcium phosphate containing colloid in the circulation for long periods of time.

d. Diffusible calcium. Some 50 to 60 per cent of the usual 10 to 11 mgm. of calcium in 100 ml. of serum is diffusible through the membranes used in the partition by ultrafiltration or dialysis. Ordinarily, it would seem reasonable to assume that in this colloid-free water clear liquid, the calcium was present simply as soluble calcium ion. However, actually, the subject of the homogeneity of the diffusible calcium has led to a great amount of controversy. Much of this centers around the attempts to find an hypothesis to explain the action of the hormone of the parathyroid gland.

It has been postulated that there is present in biological fluids, besides calcium ion, a citrate-like negatively charged complex ion of calcium or alternatively a slightly ionized or complex ion formed with the inorganic phosphate of the serum. Klinke has compiled a formidable list of evidence in favor of the organic citrate-like complex. As a proof for the existence of the phosphate complex, Benjamin and Hess have offered the results of adsorption experiments with $BaSO_4$, which, in their eyes, demonstrate that calcium ion as such is not adsorbed by

the $BaSO_4$, but that a specifically adsorbable calcium phosphorus compound is present in serum.

D. M. and L. D. Greenberg, from a critical examination of the proposed evidence and from further work of their own, arrived at the conclusion that no strong positive evidence exists for the presence of an organic citrate-like calcium compound.

In reviewing the work on the nature of the diffusible serum calcium, it is desirable to take up first the evidence dealing with the subject of the calcium ion concentration. To a very considerable extent, the validity of the proposed alternative forms of calcium rests upon correctness of the widely prevalent view that the amount of ionic calcium is only $\frac{1}{3}$ to $\frac{1}{2}$ of the amount of diffusible serum calcium. The calcium ion concentration of the blood has been estimated by electrode measurements, by calculation from the solubility of a number of slightly soluble calcium salts, and by calibrating the response of the isolated frog heart. To most of these methods, objections of one kind or another have been raised.

Neuhausen and Marshall attempted the measurement with a flowing calcium-amalgam electrode. Investigations on this electrode since then, particularly by Fosbinder, have shown that, while it can be employed in dilute solutions of calcium salts alone, in the presence of even small amounts of other cations, either above or below that of calcium in the electro-chemical series, or in solutions containing protein, the potential is at once lowered, yielding impossible results. It seems very unlikely then that the results of Neuhausen and Marshall are of any value. With the third order electrode, introduced by Corten and Estermann, Zn/ZnC_2O_4, CaC_2O_4– Ca^{++}, Corten reports that he obtained an average value of about 2 mgm. per cent as the calcium ion content of serum. The validity of this particular electrode has also been questioned. Le Blanc and Harnapp were unable to confirm the claim that this electrode gives constant and reproducible results. The experience in our laboratory has been that erratic values are obtained even with solutions of simple calcium salts. In following the causes of the potential drift, Le Blanc and Harnapp found this electrode completely unsuitable for use with biological fluids because 1, a very long time (7 weeks in the test) is required to reach a steady value, and 2, during the establishing of equilibrium a loss of calcium from the solution takes place with an equivalent replacement of zinc.

A variety of attempts to estimate the calcium ion activity are based on calculations from the solubility of such salts as $CaCO_3$, CaC_2O_4,

$Ca_3(PO_4)_2$, and $CaHPO_4$. This method of approach is thermodynamically thoroughly sound, for, as stated by Lewis and Randall, "When, at a given temperature, a solid salt is in equilibrium with a solution, the activity of that salt in the solution is fixed. It cannot be changed by any change in the nature of the solution such as would be produced by the addition of other electrolytes."

By appropriate experiments, then, solubility measurements offer a sound way of determining the calcium ion activity of biological fluids. However, if the calculations are to be valid, it is essential that a true saturation equilibrium be established between the solution and a solid phase of known composition. Proof of this criterion has never been conclusively established in any of the work so far carried out.

Rona and Takahashi proposed to calculate the calcium ion concentration from the equation,

$$[Ca^{++}] = 350 \times \frac{[H^+]}{[HCO_3]} \text{ mols per liter at } 18°.$$

The value of the constant in the equation was determined from a study of the stoichiometric solubility at 18° of simple salt solutions containing calcium and bicarbonate ions. If this equation has any validity when applied to body fluids at body temperature, it is only coincidental. E. J. Warburg has treated the equation from the point of view of the Bjerrum activity theory of electrolytes, arriving at the relation,

$$[Ca^{++}] = 307 \frac{\alpha H^+}{[HCO_3^-]} \text{ mols per liter at } 38°.$$

It is quite likely that even the corrections of Warburg do not adequately take into account the influence of the blood proteins on the ion activity.

The special interest in this equation lies in its supposed ability to indicate the change in calcium ion content with the change of pH and bicarbonate concentration of the blood. For this purpose it has been widely used, particularly in European clinical circles, to draw deductions regarding the state of the calcium in such pathological conditions as tetany, rickets, epilepsy, etc. In normal serum the corrected equation leads to the value of about 2.0 mgm. per cent as the calcium ion content.

The serious objection to this equation, which has seldom been raised, in spite of the fact that it was mentioned in the original work of Rona and Takahashi, is the unlikelihood that the blood is ever in saturation equilibrium with solid calcium carbonate. Unless this condition is fulfilled, the application of the equation is invalid. The doubt of such

an equilibrium experimentally follows from the work of Hastings, Murray, and Sendroy, who found that serum, when shaken with solid $CaCO_3$, shows no alteration in calcium content as long as inorganic phosphate is present, even when the calcium of the original serum is considerably augmented or depleted. The circulating blood, which is not even in contact with calcium carbonate as a solid phase, would seem even less likely to be normally saturated with respect to this salt. Brinkman and Van Dam attempted to employ the solubility of CaC_2O_4 for this purpose by adding increasing amounts of a soluble oxalate to a series of tubes containing the blood serum under test and using the first development of turbidity as a measurement of saturation. They used the solubility constant of 0.056, expressed in milli-mols, in the equation,

$$[Ca^{++}]\,[C_2O_4^{=}] = K_s.$$

Schulten, however, has shown that the Brinkman and Van Dam method produces supersaturated solutions and states that in these metastable regions it is impossible to find a constant solubility product. The solubility constant, according to him, lies well under 0.02.

The use of $Ca_3(PO_4)_2$ may also be ruled out since, according to Sendroy and Hastings, using the $Ca_3(PO_4)_2$ as the solid phase in serum causes a precipitation of $CaCO_3$ instead of calcium phosphate. It is an interesting coincidence that the above described methods have, according to their proponents, yielded approximately the same value for the calcium ion content of normal blood serum, namely, about 2 mgm. per 100 ml. Shear, Washburn and Kramer, and Shear and Kramer state that the crystalline salt, $CaHPO_4$, comes into equilibrium with both inorganic solutions of the composition of serum and blood serum itself in one or two hours' shaking. Using the solubility value of this salt to calculate the ion content in serum, they arrived at the average figure of 6.6 mgm. per cent instead of the low figure mentioned above. The work of the last investigators contains the implicit assumption of a unit activity coefficient for the calcium ion in the inorganic serum solutions which they used. This undoubtedly leads to a too high value in calculating the calcium ion concentration.

The biological method of estimating calcium ion activity from the contraction response of the excised frog heart, first applied to this specific purpose by Trendelenburg and von Goebel, is highly interesting. Trendelenburg and von Goebel actually did not use the method to estimate the calcium ion concentration of serum. They merely showed that there is a reduction of the level of calcium in the serum of animals in

tetany which can be corrected by the addition of 3 to 4 mgm. of calcium per 100 ml., and that a similar reduction is brought about by the slow injection of sodium oxalate solution in animals.

This method has been used only recently to estimate the concentration of calcium ions in biological fluids by McLean and Hastings. These authors arrived at figures of about 4.4 to 6.4 mgm. per cent as the calcium ion concentration in human and dog serum, in cerebrospinal fluid, and in ascitic fluid.

From the review given, it is readily seen that there is no agreement nor any valid evidence for the widespread statements that only some $\frac{1}{3}$ to $\frac{1}{2}$ of the diffusible serum calcium is ionic. The higher and the more probable estimations of the calcium ion content leave little room for other special forms of calcium in the diffusible fraction of the serum.

The hypothesis for the existence of a citrate-like compound, while resting in part upon the supposed low value of the ionic calcium, also has the assumed backing of a considerable body of experimental observations. The evidence which has been offered is based on certain electrical transference experiments and equilibration studies on serum and serum ultrafiltrates with such solids as $BaSO_4$, $Ca_3(PO_4)_2$, $MgNH_4PO_4$, and bone. In the electrical transference experiments cited, Bernhard and Beaver electrodialyzed serum through collodion and parchment membranes and demonstrated the presence of calcium in the anode compartment in 7 out of 26 experiments. Klinke also carried out a number of transference experiments. In these he found that the calcium of a serum ultrafiltrate migrated to an almost equal extent to the anode and the cathode compartments, whereas in a similar experiment with an artificial serum solution, some 80 per cent of the calcium migrated to the cathode and 20 per cent to the anode. Reversible electrodes were not used by Klinke. One of the writers and L. D. Greenberg were unable to confirm these migration experiments, although they used electrodialysis under much better controlled conditions than were employed by Bernhard and Beaver, namely, the anode compartment was not allowed to become acid. No detectable anodic migration of calcium was observed in ordinary blood sera, or in the serum of an animal which had received parathyroid extract, or in the ultrafiltrates of sera. On the other hand, when citrate was added to the sera or their ultrafiltrates, an anodic migration of calcium was very readily obtained. Similarly, the migration of the phosphate was found to be quite independent of the calcium.

As a further important line of evidence there is offered the claim that

calcium is removed from serum by positive adsorbents such as $Ca_3(PO_4)_2$, bone, $BaSO_4$, and $MgNH_4PO_4$, but not by the negative adsorbents, kaolin and animal charcoal. These claims too can be readily shown to be without foundation. In the first instance, $BaSO_4$ will remove calcium from inorganic solutions containing no organic compounds, as has been demonstrated by Benjamin and Hess and Greenberg and Larson, and secondly, properly prepared samples of the negative adsorbents, kaolin and permutit, will readily adsorb calcium from inorganic solutions, serum, or serum ultrafiltrates. Moreover, for two of the solids, bone and $Ca_3(PO_4)_2$, it may be strongly doubted that the reaction involved is an adsorption, since these, when equilibrated with solutions lacking in calcium or inorganic phosphate, produce alterations in the content of calcium and phosphate which is indicative of a solubility saturation.

From what has been given, it appears that the claims for a citrate-like calcium compound are not particularly strong. The calcium phosphorus complex, postulated by Benjamin and Hess, also has a certain amount of historical background. To explain the presumably low calcium ion content of the serum, it has been suggested from time to time that the salts, $CaHPO_4$ and $Ca(HCO_3)_2$ are only slightly ionized in solution.

The main arguments advanced for the existence of this peculiar substance are:

1. Adsorption of calcium by $BaSO_4$ does not take place from solutions of such simple salts as the chloride, phosphate, and gluconate of calcium. Adsorption of calcium takes place in the presence of phosphate when the solution is buffered at an alkaline reaction.

2. Barium sulfate removes an approximately constant amount of calcium and phosphate from normal serum or serum ultrafiltrates, leaving a residue in the ultrafiltrate which agrees with the low values found in the literature for the amount of ionic calcium of the serum.

However, quite another interpretation can be put upon the experimental observations of Benjamin and Hess. The analysis which has already been given shows that no great weight can be attached to such low figures as 2 mgm. per cent for the amount of ionized calcium present in blood serum. Further, Greenberg and Larson have shown that all $BaSO_4$ powders do not show the same adsorptive activity, and that it is readily possible to make preparations that will remove calcium almost completely from serum solutions and also from dilute solutions of any soluble calcium salt. The $BaSO_4$ of Benjamin and Hess owes its partic-

ular properties to the fact that it was prepared in an acid solution, thereby acquiring such a high positive charge through the adsorption of a film of hydrogen ions that only under very special circumstances is it able to adsorb calcium ions. One way whereby it becomes able to do this is by the adsorption of certain anions which serve to reverse the sign of the charge. Apparently, at least, a divalent ion is required. For this reason, primary phosphate will not serve. On the other hand, secondary phosphate is excellently adsorbed and so accounts for the adsorption of the calcium obtained in serum and artificial serum solutions. However, that phosphate ion is not unique in this respect is shown by the experiments of Greenberg and Larson with sulfate ion which also aids the adsorption of calcium when it is present in the solution in a manner similar to the phosphate.

The importance of the electric charge is proved by the electro endosmose measurements of Michaelis and Kodama. From these the authors showed that the charge of $BaSO_4$, which is normally positive and remains unaltered in the presence of univalent anions, becomes reversed to negative by the salts of bivalent ions such as sulfate, oxalate, and chromate. Greenberg and Larson by similar tests found that the adsorbent of Benjamin and Hess changes from positive to negative in the presence of small amounts of secondary phosphate and of sulfate ions.

The discussion which has been given shows that a very large part of the evidence offered for the existence of calcium compounds other than ordinary calcium ion in the diffusible fraction of blood serum is either insufficient, incorrectly interpreted, or, in many instances, quite incorrect. This statement applies both to the organic citrate-like compound and the special non-ionized compounds of calcium phosphate. It is true that the analysis does not categorically dismiss the possibility of such special calcium compounds, but rather shows that their existence is highly doubtful. However, until such compounds, if they exist, are firmly established, it is more useful to accept the existence of only calcium and phosphate ions in the diffusible component of blood serum. Even if low values of calcium ion activity in serum are admitted, it is qualitatively to be ascribed to an explanation familiar in connection with modern theories of strong electrolytes, namely, the result of the depressing action of the interionic influence of the electrolytes and proteins of the blood.

e. Physical chemistry of magnesium. As in many other respects, only a scanty amount of information is available on the physical chemical

state of the blood magnesium. The published work so far has dealt only with the state of the magnesium in the serum. Experiments under way in this laboratory have indicated that in laked corpuscles a considerable fraction of the magnesium is not diffusible.

The state of the magnesium in blood serum has been studied by Starry and Winternitz, Watchorn and McCance, and Greene and Powers. Starry and Winternitz first showed that some 70 per cent of the serum magnesium was diffusible by the method of compensation dialysis. The values found by the other investigators on a much larger series of animals are shown in tables 5 and 6. In human blood, by ultrafiltration, Watchorn and McCance found that the diffusibility of serum magnesium varies between 60 and 90 per cent with an average of 80 $\pm$7.0. A comparison of the results of vivi-diffusion and those obtained by ultrafiltration on the sera of the dog shows an average diffusible magnesium by either procedure of some 70 per cent (table 6).

The non-diffusible magnesium is very likely combined with the serum proteins in a similar manner and for similar reasons that have been indicated to apply to the non-diffusible serum calcium. The amount particularly, as well as the percentage of magnesium combined with the proteins, is smaller than is found for calcium.

The one important respect in which the behavior of the non-diffusible magnesium differs from the closely related calcium is that it probably does not form a colloidal magnesium phosphate. Scholtz, it is true, claims that a colloidal magnesium phosphate is formed when the magnesium of the serum is augmented. On the contrary, Greenberg and Tufts found no evidence for the existence of such a material even when the serum magnesium was augmented as much as 10-fold either in drawn blood or by intravenous injection into the living animal. The serum phosphate, it was found in these tests, remained completely diffusible throughout, even during the highest levels of the magnesium in the serum.

f. Phosphorus. Of the phosphorus compounds, the inorganic phosphate of the blood only has so far been extensively studied with regard to its relation to the metabolism of calcium and magnesium. Since the relation of the organic phosphorus compounds to the metabolism of the alkali earth elements is only slightly known, they will be but briefly considered here. Kay and Byrom give the following list of phosphorus compounds that may be present in normal blood: 1, inorganic phosphate; 2, nucleotides of adenine and pyrimidine; 3, glycerophosphoric acid; 4, phosphoric esters that reduce Fehling's solution;

5, the phospholipids, lecithin, cephalin, and sphingomyelin; and 6, unknown phosphorus residues derived from nucleic acid. The complete analysis for all of these compounds is seldom attempted. The organic phosphorus compounds are, for the most part, present in the red corpuscles and not in the plasma.

A careful study of the change in distribution of the phosphorus compounds from birth to maturity in human subjects has been carried out by Stearns and Warweg. Some of their results are reproduced in table 8. From this table it is observed that only in newborn infants

TABLE 8

Change of the phosphorus partition in the blood with age

Average values

AGE RANGE	NUMBER OF OBSERVATIONS	CORPUSCLES	WHOLE BLOOD				SERUM				CORPUSCLES				
			Total P	Lipoid P	Inorganic P	Ester P	Total P	Lipoid P	Inorganic P	Ester P	Total P	Lipoid P	Inorganic P	Ester P	Serum Ca
		per cent													
New born	12	49.6	45.9	10.8	4.9	30.1	11.8	5.3	5.6	1.0	80.6	16.3	4.3	59.7	12.5
0– 2 mos.	6	30.1	37.2	10.5	5.3	21.3	14.9	7.7	6.6	0.5	89.0	17.0	2.3	69.8	11.3
4– 5 mos.	8	33.5	40.0	13.0	5.5	21.4	14.3	7.1	6.4	0.5	91.0	24.8	3.8	63.0	11.4
1– 2 yrs.	8	36.4	42.5	12.3	4.7	25.7	14.8	8.8	5.5	0.5	91.0	19.7	3.3	69.8	11.6
4– 6 yrs.	8	37.0	40.6	12.7	4.2	23.5	15.1	9.0	5.5	0.7	84.0	18.9	2.1	62.4	10.7
10–12 yrs.	12	38.6	38.1	12.0	4.3	21.8	14.2	8.6	5.1	0.5	74.5	17.2	3.0	54.3	10.8
12–15 yrs.	10	39.7	38.6	11.6	4.2	22.7	13.8	8.6	4.7	0.6	76.4	16.1	3.5	56.6	10.7
15–25 yrs.	21	45.3	35.4	11.2	2.9	21.2	12.2	8.2	3.4	0.6	63.5	14.8	2.4	46.1	10.8

Analytical values are expressed in milligrams of element per 100 ml.

STEARNS, G. AND E. WARWEG. J. Biol. Chem. **102**: 749, 1933.

do many of the values differ considerably from the work reported on adults. An exception to this is the inorganic serum phosphate, which is about 2 to 3 mgm. per cent higher in all normal children up to adolescence, as well as in newborn infants, than it is in the adult. There is a considerably higher content of ester phosphorus and lower lipid phosphorus in the sera of the newborn than is found at a later period of life. The distribution of the phosphorus compounds in the corpuscles of infants and older children is nearly the same as in adulthood, and an apparent difference in the composition of the whole blood of newborn infants is merely the reflection of a higher corpuscle content.

The functions of the organic phosphorus compounds, listed by Peters and Van Slyke, are:

1. All of the organic compounds form a reserve store of phosphoric acid for the animal organism. An apt illustration of this is in the developing egg in which these compounds provide the phosphorus for the formation of nucleic acid and of bone.

2. The ester phosphorus, particularly glycerol ester, may be of importance in providing inorganic phosphate for bone formation.

3. The hexose phosphates undoubtedly have an important function as the intermediate compounds in carbohydrate metabolism.

4. The phosphoric esters may perhaps be of significance in the renal excretion of phosphate (Kay, Eichholtz, Robison).

5. The phosphatides, or phospholipids, which are essentially compounds of phosphoric acid with fatty acids, it is currently believed, serve in the transport of the fatty acids throughout the body and particularly across cell membranes. They may also perhaps constitute an intermediate step in preparing the fats for combustion. In the exercise of the function of transport, the phospholipids exchange their fatty acids or even undergo complete hydrolysis, e.g., in the formation of milk fat. The corpuscles contain between 2 to 3 times as much of this form of phosphorus as does the plasma.

6. *Cephalin*, one of the phospholipids, appears to be essential for the formation of thrombin and the coagulation of the blood.

7. Organic phosphorus compounds form essential integral parts of the structure of cells. Nucleic acid may be cited as an example. Tung has recently shown that about 50 per cent of the material in the stroma of the red corpuscle consists of lipids, probably largely phospholipids.

8. Organic phosphorus compounds act as non-diffusible anions in the cells and contribute toward the neutralization of the alkali. Each of the compounds of this sort which has been isolated has at least one acid hydrogen free and capable of combining with alkali.[6] In this manner they contribute to the buffering capacity of the body. Because they are non-diffusible they also take part in building up the unequal distribution of the diffusible ions between cells and plasma resulting from the Donnan principle.

In the body the inorganic phosphates are in two forms, the solid

[6] According to unpublished work of Jukes from this laboratory, lecithin shows no acid or basic properties, and therefore does not contribute to the buffering action of the blood. This compound, as well as other phospholipids such as cephalin which show acid properties, must be regarded as zwitterions.

calcium phosphate of the skeleton, and the dissolved phosphates of the body fluid. The skeletal composition is considered elsewhere. Three-fourths of the total phosphorus of the adult body is in the skeleton.

The soluble inorganic phosphates of the body fluids have been studied chiefly with respect to the blood serum. The concentration varies between 3 and 9 mgm. per 100 ml. This phosphate exists as a mixture of the primary and secondary phosphate ions, the exact proportions being determined by the pH of the media. At pH 7.4, the ratio of secondary to primary phosphate is about 4:1. The dissolved inorganic phosphate functions 1, in the maintenance of the acid base equilibrium; 2, in the metabolism of the alkali earth elements, and 3, in the metabolism of carbohydrates.

The immediate buffering value of the blood phosphates is not great because of the small amount present. However, the cumulative effect as a vehicle of acid excretion by the kidney is important.

3. PHYSIOLOGICAL AND PATHOLOGICAL VARIATION IN THE CALCIUM, MAGNESIUM AND INORGANIC PHOSPHATE OF THE BLOOD. *a. Normal variation.* The range of variation in the serum calcium of healthy human subjects, it has already been mentioned, is between 9.0 and 11.5 mgm. per cent. This relationship is only slightly affected by age and is apparently independent of sex. In newborn infants, a higher serum calcium value has been reported. Bakwin and Bakwin give 11.0 $\pm$0.9 as the average of 300 cases, and Stearns and Warweg report the even higher average value of 12.5 mgm. per cent. This augmented figure subsides to nearly normal limits within the first few months of life. The placental serum calcium has nearly the same value as is found for the newborn (11.3 $\pm$0.9 mgm. per cent), but the maternal blood, interestingly enough, has at this time a considerably lowered calcium value (9.8 $\pm$0.9 mgm. per cent). In extreme old age, around 80 years, Gresheimer and Johnson report a decrease of about a milligram in the average level of the serum calcium.

The heavy drain upon the minerals of the maternal organism during pregnancy might well be expected to induce marked effects. In spite of this, the calcium concentration remains constant until nearly the last month of pregnancy, and then it shows only an average lowering of about 1 mgm. per cent. At the same time, the concentration of the serum proteins and of the inorganic phosphate also falls slightly. After parturition, these substances are rapidly restored to normal. It has been suggested that pituitary activity at the close of pregnancy is responsible for the lowering of the calcium level.

In the adult human, the serum calcium is nearly equally divided into diffusible and non-diffusible components. A nearly similar distribution was found by Needels and Marberg in the blood of 422 children between the ages of 10 and 17. The average value for diffusible calcium of all of their groups is about 55 per cent of the total, and the percentage in any group fluctuates only slightly from this figure.

The serum calcium values of a large number of dissimilar animal species fall within the limits found for man. Some figures illustrating this are given in tables 6 and 7. According to Palmer, Gortner and Rude, the average serum calcium content of cattle is 12.9 $\pm$1.7 mgm. per cent. This value is considerably higher than is reported by other investigators. On the other hand, the results of Sjollema and Seekles (average 9.4) seem a little low for normal animals. However, this correspondence with the content of calcium in human sera is by no means true of all animals. The most striking mammalian exception to our knowledge is the rabbit. The pig too has a high serum calcium with a reported average of 12 mgm. per cent. Harnes reports the range of serum calcium in the rabbit as being between 14.5 and 18.5 mgm. per cent with a mean of 15.5. Somewhat lower figures have been reported by others. Diet, it has been found, very considerably influences this calcium level in the rabbit. It has been noted that cabbage, when fed to rabbits, especially increases the serum calcium content. Bran and oats are said by Dupre and Semeonoff to have just the opposite action. Culhane is of the opinion that a specific calcium raising principle in cabbage is responsible for this increase. Kapsinow and Underhill deny the existence of such a factor. The serum calcium of the rabbit is subject to much greater normal fluctuations than are found in other animals.

Smith has analyzed the composition of the body fluids of various turtles and sharks. The serum calcium in these animals was found to lie between 12.5 and 26.0, magnesium between 1.2 and 19, and inorganic phosphate between 2.8 and 11 mgm. per cent. Another illustration of an unusual level of serum calcium is found in birds, the most studied of which has been the domestic fowl. In male birds or non-laying females, the calcium value lies within the range or only a little above the results reported for man (see table 7). However, as was first shown by Riddle and Reinhart, at the onset of the laying period, the serum calcium of the hen increases to such values as 20 to 30 mgm. per cent. Quite evidently this change is in some way connected with the endocrine secretions of the sex organs. This matter will be discussed further in

another section. The underlying cause of the high calcium value in rabbits is more obscure.

Although the total serum calcium levels are nearly the same in most animals, differences may appear in the distribution of diffusible and non-diffusible calcium when this is carefully examined. The only instance of a sufficiently extensive and controlled series which brings this out is shown in the comparison between the values of normal human and dog blood which is shown in tables 5 and 6. The data show that with nearly the same variability of total serum calcium, human serum contains an average of 53 ±5 per cent of diffusible calcium, while in the dog it is about 62 ±6 per cent diffusible. This is a species distinction which would be unsuspected from a mere total serum calcium analysis. The greater amount of diffusible calcium in the serum of the dog naturally implies a lower amount of non-diffusible calcium. From the previous discussion on the relation between non-diffusible calcium and serum proteins, particularly albumin, some connection might be expected to follow with the respective protein content in the blood of these animals. Unfortunately, complete protein analyses are not available for comparison on the same humans and dogs used for the calcium studies.

The distribution of calcium in the serum of animals with normally high calcium values is also exceedingly interesting. The figures taken from Updegraff, Greenberg, and Clark show that the greater part of the high serum calcium values of the rabbit is in the diffusible fraction, the average value being about 67 ±6 per cent. The non-diffusible calcium (4.2 ±0.8 mgm. per cent) is about the same as in the dog. The observation that the serum inorganic phosphate in this animal appears to be completely diffusible is in keeping with this. On the other hand, when the serum calcium increases in the laying hen, the increase is in the non-diffusible calcium fraction. From the results of Laskowski, the diffusible calcium remains the same as is found in the non-laying hen. At the same time, the diffusibility of the phosphate is greatly reduced, indicating the formation of colloidal calcium phosphate. The amount of colloidal calcium phosphate which can be calculated to be formed is not sufficient to account for all of the increase in the non-diffusible calcium of the laying hen. Apparently, some other form of non-diffusible calcium is also involved.

In contrast to calcium, magnesium is present in the blood corpuscles as well as in the plasma, the greater amount being in the corpuscles. Moreover, the percentage variation in the magnesium content of the

blood under normal conditions, both in plasma and corpuscles, is much wider than is found with calcium.

Most of the analytical work carried out on this element has been done by precipitating it as magnesium ammonium phosphate and then determining colorimetrically the phosphate carried down. Many modifications of this procedure have been published. Recently, analysis with 8-hydroxyquinoline, which was introduced as an analytical agent by Berg, has been adapted to blood.

In the hands of Greenberg, Lucia, Mackey and Tufts, the latter method has revealed the presence of a good deal more magnesium in the red corpuscles than has hitherto been suspected. The content of this element in the serum and in the oxalated plasma of the same blood, it has been found, is identical.

It is commonly stated that in health the magnesium in human sera varies between 2 and 3 mgm. per cent. Greenberg et al. (table 5) observed a range of 2 to 3.6 mgm. per cent with a mean of 2.7 $\pm$0.3 in plasma. Watchorn and McCance hold that the widest limits of variation in health are between 2.3 and 2.66 mgm. per cent. Undoubtedly this range is too restricted, probably because the series was selected from a restricted population (laboratory associates) of closely the same age.

Until recently, the amount of corpuscular magnesium was practically an unknown quantity. Abderhalden reports about 1 mgm. per 100 grams of corpuscles in the blood of the cow and sheep, more in other species, with a maximum of 9.0 mgm. in the pig. Most of his figures are probably too low. Greenberg, Lucia, Mackey and Tufts found a variation of 5.4 to 7.8 mgm. per 100 ml. of corpuscles in young human adults with a mean of 6.6 $\pm$0.5. In dogs a variation of 7 to 10.5 mgm. per 100 ml. of corpuscles has been found with a mean value of 8.0 (table 6). In human pathological cases, particularly those associated with anemia, the amount of magnesium in the red corpuscles may increase to as much as 10 to 15 mgm. per 100 ml. of corpuscles. As with the plasma magnesium no association has so far been made in this variation in the corpuscles with any specific pathological condition. The published data are not sufficiently extensive to determine whether certain of the common mammalian species are characterized by unusual magnesium levels in the blood, such as has been noted in connection with the serum calcium. This may perhaps be due to the much greater variation which prevails in the blood magnesium of all animals.

b. Inanition. As has already been pointed out, the calcium content

of the blood serum is ordinarily quite constant, that of the magnesium and inorganic phosphate much less so. The factors that determine the level of the calcium as well as of the inorganic phosphate and the magnesium are still imperfectly understood. Calcium and phosphate levels are strongly influenced by vitamin D and the parathyroid hormone. Sufficient evidence exists to show that there is a mutual influence of the three ions upon each other, particularly between the calcium and the phosphate. The level of the plasma proteins, too, exerts a marked effect upon the calcium content.

The composition of the blood with respect to these three substances in the last analysis must depend upon a balance between their absorption from the ingested food, their excretion by way of urine and feces, and their storage and release from the tissues, more particularly the skeleton of the body. Ordinarily, the relation between storage and release from the skeleton so far preponderates in maintaining the composition of the milieu, that the influence of absorption and excretion is observed only in extreme upsets. The transport of calcium, magnesium, and phosphorus from food to body tissues and from tissues to the excretory organs must necessarily take place through the blood stream, but it is difficult to obtain evidence of this, particularly for the first two mentioned elements, by means of fluctuations of their level in the blood.

A continuous supply from the food is not essential in order to maintain the serum calcium, magnesium, or inorganic phosphate level. During long periods of starvation in animals, the calcium is maintained quite constant almost until the death of the animal. The same is true for the magnesium and inorganic phosphate. With dogs and cats, Morgulis et al. found that the serum content of these elements does not change appreciably until the fasting has progressed beyond a 20 per cent loss in weight. With a 40 per cent loss of weight, the average decrease in calcium is about 15 per cent. The inorganic phosphate level is reduced about 10 per cent. On again feeding the animals there is observed a rapid return to the pre-fasting level. In human subjects, Farquharson and Tibbits observed almost no variation during short periods of fasting.

A somewhat different problem that has been investigated is the possible existence of diurnal rhythms in the level of these elements. According to Nicolaysen, the calcium level may remain fairly constant; however, variations of as much as 2 mgm. per cent may be found in the same individual from one day to another. On an ordinary diet, Farquharson and Tibbits found no daily variation. A large amount of fat in

the diet produced in one of the subjects an increase from 9.6 to 10.8 mgm. per cent, and a fall in the inorganic phosphate from 3.9 to 2.3 mgm. per cent. High protein (200 gm. daily) in one instance increased the amount of calcium from 9.6 to 11.4 mgm. per cent. Greenberg and Gunther observed irregular variations in man and dogs amounting to as much as 1 mgm. per cent during short periods of inanition.

c. Transport. Even after the ingestion of food containing considerable amounts of calcium and magnesium, during which time there is known to be a considerable absorption of these elements, there are produced only negligible changes in the blood composition of these substances. This is due to slow absorption. Only following the postprandial ingestion of soluble calcium salts, the injection of these salts, or by treatment with large doses of parathyroid extract or of vitamin D, is the exchange and transport of sufficient magnitude to produce alterations in the blood composition. The transport of the blood phosphorus, on the other hand, is more readily demonstrable. This, it appears, is due to the relation of this element to the metabolism of fats and carbohydrates in the animal body. As long ago as 1915, Bloor showed that there is an increase in the phospholipid content paralleling the increase in the blood fat, following the ingestion of fat-containing food. Most of this increase appears in the blood corpuscles. A similar relationship is found in the lipemia of starvation. Phosphatides are believed to play a preponderant rôle in the transfer of fats across cell membranes. This is significantly illustrated by the work of Meigs, Blatherwick, and Cary on the accumulation of fat during milk secretion. Instead of the neutral fat of the blood being the source of the milk fat, these authors showed that the active mammary gland of the cow receives phospholipids from the blood stream, and the fatty fraction therefrom is converted into milk fat. The residual phosphorus is partly retained in the milk and partly returned to the blood as inorganic phosphate.

Another important example is in connection with the absorption of fat from the intestines. The iodine numbers of the fatty acids of the phospholipid of the intestinal mucosa of cats which had recently been fed such unusual fats as cod liver oil or cocoanut oil, have been shown by Sinclair to be higher than those of cats receiving ordinary fat in their diet. The concentration of phosphatides in the intestinal mucosa, however, remains unchanged. The inference to be drawn from this is that fatty acids from digested fat displace fatty acids which were already combined in the phospholipids of the mucosa cells. Conse-

quently, it would appear to follow that the phosphorus of these phosphatids assists in the transport of fat across the intestinal mucosa into the lymphatics. Fluctuations of the phosphorus, particularly the inorganic phosphate of the blood, also occur in association with metabolic transformations of the carbohydrates. This will be brought up again in another connection.

d. Relation of the serum proteins and phosphate to calcium level. The level of the serum proteins, it is well known, contributes toward the height of the serum calcium. The basis of this, as has already been brought out, is the combination of a part of the calcium with proteins, probably chiefly the albumin, to form the non-diffusible part of the calcium. Practically, this is a factor of great importance. Salvesen and Linder have pointed out that, although in severe cases of Bright's disease and of heart failure the serum calcium level may be much reduced, none of the symptoms associated with the low calcium level of parathyroid deficiency are observed. Furthermore, the decrease in the amount of serum calcium in cases of Bright's disease without phosphate retention parallels the reduction in the content of serum proteins. When the protein concentration is again raised, it is found that the calcium likewise increases. The association of a lowered serum calcium value with a reduction in the level of the proteins has been noted in jaundice and neoplastic conditions. It probably also occurs in protein deficiency resulting from protein starvation. On the other hand, following parathyroidectomy, the concentration of calcium becomes reduced, despite the fact that the plasma proteins remain constant.

For a long time the belief has existed that calcium and the inorganic phosphate of the serum are reciprocally related in such a way that an increase in phosphate results in a reduction in calcium and vice versa. Historically, a part of this belief is predicated on the observations of Binger that injections of large amounts of neutral or alkaline phosphate reduced the serum calcium and the reduction resulted in tetanic symptoms. When amounts of inorganic phosphate equivalent to 150 mgm. of phosphorus per kilo are injected, the blood calcium is reduced to about 6 mgm. per cent and tetanic seizures are said to supervene. With acid phosphate or phosphoric acid in similar doses, the calcium is also reduced about an equivalent amount, but with no tetanic effects. Tisdall confirmed the reduction in the calcium produced by phosphate, but did not observe any generalized symptoms of tetany.

Greenwald has criticized this work because of the extensive blood dilution produced by the large volume of fluids injected during these

experiments. In similar experiments carried out by him no tetany was observed. He states that there may be an occasional twitch of a single muscle, but never a condition with the generalized convulsions observed in the tetany associated with a parathyroid deficiency. Greenwald observed that Na_2SO_4 injections produced quite as marked decreases in serum calcium as did phosphate, and, most interestingly, injections of NaCl, which did not result in a lowering of the calcium level, produced the most pronounced muscular twitchings and convulsions of any of the above salts. In this author's estimation, the symptoms observed were probably due largely to upsets in both osmotic concentration and to changes in pH due to selective retention of the cations.

However, that there is a mutual influence between calcium and phosphate in the blood seems likely in spite of this criticism. Greenwald does not deny this and states, "Since practically all of the calcium in the body is present as calcium phosphate, obviously any disturbance in calcium metabolism must affect the phosphate, but there may be changes in the latter that have no direct relation to the former."

Some of the observations favoring a reciprocal relationship between calcium and inorganic phosphate will now be discussed. In cases of nephritis, where there is a retention of phosphate even though there is little reduction in the amount of serum proteins, the calcium level is prone to be low. An analysis of the data published by Schmitz, Rohdenburg and Myers, given in table 9, illustrates this point. In some 58 observations the mean serum calcium concentration was calculated to be 8.2 as compared to 10.3 mgm. per cent for normal human individuals. The mean content of serum phosphate was 8.5, contrasted to 4.0 mgm. per cent for normal subjects. These data certainly favor the view that an increase in phosphate content goes hand in hand with a reduction in the level of calcium. This reduction in the amount of serum calcium is particularly marked in cases with uremia. De Wesselow, Mainzer and others have suggested that the motor irritability, the tremor, and the muscular twitchings in uremia are referable to a deficiency in the blood calcium. However, there is no evidence that the ordinary uremic motor disturbances and convulsions are attended by the alterations in the electrical excitability of the neuromuscular system which are characteristic of tetany.

The opposite side of the picture is to be observed in the hypercalcemia of hyperparathyroidism, both natural and experimental. From the data collected by Barr on the blood picture in hyperparathyroidism, the calcium ranges between 10.0 and 23.6 mgm. per cent with an average

TABLE 9

Composition of the blood in certain pathological conditions involving a disturbance in the alkali earth elements and in phosphorus

	NUMBER OF OBSERVATIONS	RANGE*	MEAN	SOURCE OF DATA
(a) Rickets (Human subjects)				
Serum calcium	60	7.0–13.0	9.0	(1)
Inorganic phosphate	60	0.6– 7.0	3.0	(1)
Serum magnesium		1.8– 2.5	2.2	(1)
(b) Osteomalacia (with tetany) (Human subjects)				
Serum calcium	10	5.0– 7.4	6.0	(2)
Inorganic phosphate	10	1.8– 3.8	2.7	(2)
Magnesium		1.8– 3.8	2.7	(2)
(c) Human parathyroid tetany, juvenile tetany				
Serum calcium	30	6.0–10.5	7.9	(3, 4)
Diffusible calcium	22	2.4– 5.2	3.5	(3, 4)
Inorganic phosphate	9	2.0– 8.0	5.0	(3, 4)
Serum magnesium			2.0	(3, 4)
(d) Parathyroid tetany (Dog)				
Serum calcium	25	4.0–10.0	7.0	(5)
Diffusible calcium	12	2.0– 7.0	4.4	(6)
Inorganic phosphate	11	5.0–11.0	7.0	(6)
(e) Hyperparathyroidism (Human subjects)				
Serum calcium	24	10.0–23.6	15.9	(2)
Inorganic phosphate	24	1.0– 5.0	2.2	(2)
(f) Chronic glomerular nephritis (Human subjects)				
Serum calcium	58	3.0–13.0	8.2	(7)
Serum magnesium	17	1.3– 7.9	3.0	(8)
Inorganic phosphate	94	3.0–25.5	8.5	(7)
CO_2 volume, per cent	71	20.0–80.0	44	(7)
Albumin, per cent	6	1.5– 2.9	2.4	(8)
Globulin, per cent	6	1.7– 3.3	2.7	(8)

TABLE 9—*Concluded*

	NUMBER OF OBSERVATIONS	RANGE*	MEAN	SOURCE OF DATA
(g) Nephrosis (Human subjects)				
Serum calcium	11	7.4– 9.5	8.0	(8, 9)
Diffusible calcium	3	4.8– 5.7	5.4	(9)
Inorganic phosphate	8	3.2– 5.8	4.7	(8)
Serum magnesium	4	1.5– 2.5	2.0	(8)
Albumin, per cent	10	0.9– 2.9	1.9	(8, 9)
Globulin, per cent	10	2.1– 3.5	3.0	(8, 9)

* Analytical figures for inorganic substances are expressed in milligrams of element per 100 ml.

(1) Howland, J. and B. Kramer. Am. J. Dis. Child. **22**: 105, 1921.
(2) Barr, D. P. Am. J. Cancer. **16**: 1421, 1933.
(3) Gunther, L. and D. M. Greenberg. Arch. Int. Med. **47**: 660, 1934.
(4) Liu, S. H. J. Clin. Invest. **5**: 259, 285, 1927.
(5) Cameron, A. T. and V. H. K. Moorhouse. J. Biol. Chem. **63**: 687, 1925.
(6) Reed, C. I. J. Biol. Chem. **77**: 547, 1928.
(7) Schmitz, H. W., E. L. Rohdenberg and V. C. Myers. Arch. Int. Med. **37**: 233, 1926.
(8) Salvesen, H. A. and G. C. Linder. J. Biol. Chem. **58**: 617, 1924.
(9) Greenberg, D. M. Unpublished data.

of 15.9 mgm. The serum phosphate in these cases varied between 1.0 and 5.0 mgm. per cent with the much lower than normal average of 2.2. Albright, Bauer, Cockrill, and Ellsworth, in an examination of 150 analyses of 2 cases of parathyroid tetany and of 11 other cases receiving parathormone, observed a marked inverse relationship between the calcium and the phosphate.

Considering each case individually, the product of calcium and phosphate, expressed as mgm. per cent of calcium and phosphorus respectively, tended to be a constant. In a case of hypoparathyroidism, this product appeared to be 60.4, so that when the calcium was 5.0 mgm. per cent, the phosphorus was 10.7 mgm. per cent. Under parathormone therapy, increasing the calcium to 10.5 reduced the phosphorus to 5.4 mgm. per cent. When the calcium once more fell to 7.4, the phosphorus promptly increased to 8.7 mgm. per cent.

The curve for the relationship of $Ca \times P = \text{constant}$, as noted by Albright et al., is a rectangular hyperbola. The significance of this is that at low calcium concentrations, the change in phosphate must be

quite large to maintain the constant, and only little at high calcium levels. However, from subject to subject, the constant was different, apparently being unique for each individual. For all the subjects the average product was 36.1.

In animal experimentation similar observations have been made. In dogs following parathyroidectomy, there is some increase in inorganic phosphate along with the observed reduction in the content of calcium. Normal dogs in which a rise in the amount of serum calcium is induced by moderate doses of parathyroid extract show a concomitant drop in the amount of inorganic phosphate provided that the phosphate level is initially 4.0 mgm. per cent or above. Greenberg and Gunther noted that there was no drop in the amount of phosphate if the initial value is about 4 mgm. per cent of phosphorus or less.

The explanation tentatively proposed by Albright et al. for this inverse relationship and which is in the background of other work in this field is that the inverse relationship is dependent upon the saturation level of some calcium phosphate salt, exactly which one being undefined. Of the salts of calcium and phosphoric acid, only $CaHPO_4$ has a solubility which would approximately fit in with this conception. Since this salt does not appear to be present in bone, it is commonly rejected as the determinant of the solubility of the calcium and phosphate in the blood. There are other reasons for doubting that the relations observed are dependent upon an actual solubility. The different products found from individual to individual are against this idea. When the phosphate level is reduced by means of glucose and insulin, the rise in calcium, if it takes place at all, is far too slight to be fitted into the scheme. The observations on day to day variation in calcium and phosphate do not then fit the theory, and the variation in the blood of healthy infants shows no constant relationship between the level of serum calcium and inorganic phosphate or of the serum proteins. The lowering of the blood calcium which is observed to follow the injection of inorganic phosphate has led a number of investigators to suggest its use as a therapeutic agency in the treatment of conditions associated with an hypercalcemia. Since increasing the blood inorganic phosphate level in the presence of a high concentration of calcium favors the formation of colloidal calcium phosphate, this form of therapy may lead to harmful results.

A number of attempts have been made to express mathematically the reciprocal relations that have been discussed between the serum calcium and the levels of protein and inorganic phosphate in the blood. From a

statistical treatment of the analyses of calcium, inorganic phosphate, and protein content of the sera and edema fluid of cases in which there was no reason to suspect a direct effect on calcium metabolism, Peters and Eiserson developed the equation,

$$\text{Ca} = 7 - 0.255 \text{ phosphate} + 0.556 \text{ protein}$$

in which Ca and inorganic phosphate are expressed in milligrams and the protein in grams per 100 ml. of serum.

In the opinion of Peters and Eiserson, any abnormality of the calcium concentration can only be properly interpreted if the amounts of phosphate and protein have been simultaneously determined. A low or a high serum calcium value can be considered as evidence of an abnormality in calcium metabolism only if the phosphate and protein content are normal. As has already been mentioned, Greenwald feels that the supposed reciprocal relation between the amount of serum calcium and the inorganic phosphate is probably non-existent. He has therefore attempted to find a relationship for the serum calcium dependent only upon the content of serum proteins. From an analysis of the published data available in the literature, he arrived at the equation,

$$\text{Ca} = X + 0.875 \text{ protein}$$

in which X is a quantity which varies between 5.0 and 3.7 for different adults, and has the value of about 6.3 in the blood of infants. The value of X has to be determined separately for each analytical series.

Any number of equations can be derived, all of which will more or less give a fairly good representation of any single series of results, but so far a sufficient background of underlying knowledge is not available to derive any completely valid equation. Because the protein bound calcium probably is very largely united to the albumin rather than to the total protein, the total protein content of the serum can only give an inadequate representation. If the value of X in Greenwald's, and the constant 7.0 in Peters and Eiserson's equation have any meaning, it would appear to be as a representation of the diffusible calcium content of the serum. It is not likely that the amount of inorganic phosphate only determines the level of the diffusible calcium. For these reasons, attempts at quantitative relations cannot be highly successful in the present stage of knowledge on this subject.

e. Disturbances in calcium, magnesium and phosphorus metabolism. Disturbances in calcium and phosphorus metabolism have been postulated and looked for in many pathological conditions. Those in which this fact has definitely been established are considered elsewhere in this

review. Here we shall briefly mention a few conditions in which a connection to the metabolism of alkali earth elements has gained prominence, but in which actually the relation is highly doubtful. In the list of such diseases we may mention epilepsy, tuberculosis, neoplasms, essential hypertension, polycythemia vera, jaundice, and allergies of various types. Outside of a certain similarity of epileptic seizures to hypocalcemic tetany, no worthwhile evidence for any causal connection of calcium to epilepsy has ever been offered. Similarly, the only evidence for an involvement of calcium and phosphorus in tuberculosis appears to be the calcification which is observed in tissues attacked by this disease. Claims have been made of an abnormally high serum calcium value in polycythemia vera. Recent reliable analyses, however, do not bear this out. Upsets in the serum potassium to calcium ratio have been postulated in both arterial hypertension and in allergic conditions. In a study of hypertension, Weinstein and Weiss found no alteration from the normal in the serum calcium level, but did note a slight average increase in the potassium. They attribute this small increase in the ratio to impairment in the circulation. In allergic conditions both total serum calcium and the diffusible calcium values appear to be quite normal. A deficiency in the amount of calcium in the blood stream in jaundice has long been suspected and sought. The reason for this is the prolonged bleeding time often associated with jaundice. Lack of space forbids discussing in detail of the various aspects of this subject. However, it may be said that the most recent work rules out any deficiency in blood calcium as being involved in this condition. While there is at times a lowering in the level of blood calcium in jaundice, it seems, as is indicated by the work of Gunther and Greenberg, to be the result of a reduction in the serum protein content. Snell and Greene and Gunther and Greenberg found a normal content of diffusible calcium in jaundice. Figures from more than one hundred patients, given in the paper by Snell and Greene, indicate that the total serum calcium value in jaundice is in the majority of cases about normal.

Both calcium and magnesium have been called into question in neoplasms. A good review of the subject has been published recently by Shear. Theis and Benedict and Gunther and Greenberg have found a considerable lowering in the level of serum calcium of about 50 per cent of the patients afflicted with carcinoma and other malignant tumors. However, this too appears to be the result of a lowered protein content rather than a reflection of a deficiency in physiologically active calcium.

The idea promulgated by Delbet that a deficiency in magnesium is concerned with the incidence of cancer has been taken particularly seriously in France. Surveys have been carried out in France and Egypt attempting to show an abnormally high cancer rate in regions with soils low in magnesium and the converse condition in regions of high magnesium. It is alleged that magnesium salts inhibit cancer growth. Most of the work on magnesium in this connection, as is pointed out by Shear, is not of such a quality as to inspire any confidence in its value.

In diseases of the kidney, the plasma magnesium content is highly variable. Watchorn and McCance report values of from 1.5 to over 10 mgm. per cent. In patients with nephritis, Hirschfelder has observed levels of serum magnesium which are compatible with both tetany and anesthesia. The cases with low blood magnesium (0.9 to 1.37 mgm. per cent) had muscular twitchings or outright convulsions. The twitchings disappeared when the subjects were given magnesium sulfate orally. The amount of serum magnesium rose to between 3.3 and 5.8 mgm. per cent.

The opposite conditions were also observed by this author in nephritic patients to whom epsom salts had been administered. The blood magnesium levels of these patients were between 9.8 and 11.3 mgm. per cent. They were definitely drowsy and unresponsive. According to Hirschfelder and others, a large part of the ingested doses of epsom salts in amounts of between 20 and 30 grams is absorbed. In normal subjects the absorbed magnesium is excreted so rapidly that there is no significant rise in plasma magnesium, but in nephritic animals, such doses increase the amount of plasma magnesium even up to narcotic levels.

f. The effect of the ingestion and injection of calcium and magnesium salts. Experimental work on the influence of ingested or injected doses of salts of the alkali earth elements should contribute a great deal to the knowledge of their metabolic and physiological functions. The complexity of the results that have been obtained, however, precludes an explanation of their action on the basis of any very simple theory.

For a long time the increase in the amount of serum calcium which can be obtained after the ingestion of a soluble calcium salt was missed by investigators. This, it is now known, was due to the fact that blood samples were not taken at the proper intervals, or the partaking of food during the experimental period was permitted. The typical result which is obtained, if the salt is administered post prandially in sufficient dosage, and no emesis or diarrhea occurs, is a considerable increase in

the serum calcium level which reaches a maximum in about 2 hours and then drops back to the pre-ingestion level in about 4 hours. This sequence has been observed both in dogs and in human subjects. With the usual ingested dose, Greenberg and Gunther observed that the increased amount of serum calcium is confined practically in toto to the diffusible calcium fraction. The amount of increase in blood calcium which may be obtained is a matter of considerable dispute, with increases of between 10 and 80 per cent being claimed. Also the belief has long persisted that certain of the salts are more efficacious in inducing an increase in the blood calcium, the list usually being headed by calcium chloride. The work of Greenberg and Gunther indicates that there is no difference in efficiency in inducing a rise in calcium with any of the commonly used soluble calcium salts. The increase obtained was found to be proportional to the calcium content of the dose employed, irrespective of which salt was administered. On a total weight basis, this, of course, gives an advantage to the salts of lower molecular weight. A 2 gram dose of calcium which corresponds to about 7 grams of the chloride, 8 grams of the acetate, 11 grams of the lactate, and 22 grams of the calcium gluconate, produced in this work on the average a serum calcium increase of 1.5 mgm. per cent. This represents an augmentation of about 15 per cent.

Along with the rise in serum calcium there is usually, but not invariably, observed an increase in the level of inorganic phosphate which reaches its maximum about 6 hours after the ingestion of the calcium salt. The amount of this phosphate increase does not parallel the calcium rise and usually is about 1 mgm. per cent irrespective of the calcium dose. No increase in serum calcium was observed in human subjects following the ingestion of sweet pasteurized milk, but the same amount of acidified milk led to a rise of roughly 1 mgm. per cent in both the calcium and the inorganic phosphate value of the blood with a maximum at 8 hours after the ingestion of the milk.

Wokes has studied the rate of absorption of calcium salts in mice by the ingenious method of the antagonistic waking effect on magnesium narcosis. From this work, Wokes decided that calcium chloride is the most readily absorbed salt. If the absorption value of calcium chloride is placed at 100, then the indices of other salts are given as: mixed calcium lactate and sodium lactate 100, chlorate 70, calcium lactate alone 70, and gluconate 45.

The features observed following the injection of salts, as might be expected, differ very markedly from the ones on ingested salts. Intra-

venously injected salts of calcium, magnesium, and phosphate produce their highest peak in the blood immediately following the injection, and then continuously drop back to the initial value. The rate of fall of intravenously injected blood calcium has been studied by Greville who found that at any instant it is proportional to the excess over the normal level present in the blood at that instant. Mathematically, this can be expressed by the equation,

$$\log X = A - kt$$

in which X is the amount above the initial level, t is the time, and A and k are empirically determined constants. From the studies of Greenberg and Tufts, the decrease in the amount of serum magnesium and of inorganic phosphate following the intravenously injected salts follows the same path as is found with calcium salts. The behavior of the inorganic serum phosphate following the injection of a calcium salt is rather erratic. There commonly is found a drop in the phosphate level about the time the calcium is at its peak, and then a return to either the initial value or values even higher than the initial within about 4 hours following the injection. Occasionally an immediate rise rather than a fall in phosphate level is observed. The implication of this is that several oppositely acting processes are stimulated by the injection. The injection of calcium salts also produces an increase in the amount of plasma magnesium, the peak coming about 2 hours after the injection. The magnesium level then subsides. A similar rise in the magnesium concentration has been noted after injections of parathyroid extract, the rise appearing a few hours after the injection and many hours before the calcium increase reaches its peak. The fall in the blood calcium level following the intravenous infusion of large doses of phosphate has already been discussed. When smaller amounts are injected, in doses which can produce but little blood dilution, the serum calcium value increases 1 to 2 mgm. per cent in the first hour and then falls below the initial level by about 1 or 2 mgm. per cent. The magnesium level also undergoes a continuous decrease for a period of about 6 hours after the injection. Injection of calcium salts has a marked effect on the heart and circulation. There is produced a definite increase in blood pressure. Lieberman states that intravascular clotting sometimes follows calcium salt injection. Stransky has observed that narcotic doses of magnesium salts (0.02 to 0.03 gm. Mg. per kilo), injected subcutaneously into the rabbit, caused a marked reduction in the amount of serum calcium. Using doses of about 0.02 gram

per kilo, injected intravenously into dogs, Greenberg and Tufts observed almost no change in the level of serum calcium, but found that the inorganic phosphate level is lowered considerably.

Substances capable of interacting with the alkali earth elements or with the phosphate are also capable of producing marked alterations in their blood composition.

Soluble oxalates reduce the amount of blood calcium to the point of convulsions and death. Baudouin, Lewin, and Azérad were able by this means to reduce the serum calcium level to half its initial value. It returned to normal in about 2 hours. Jodlbauer observed a reduction in the amount of blood calcium after both oxalate and fluoride poisoning. Jacoby and Friedel also noted a decrease in the magnesium level and irregular changes in the percentage of inorganic phosphate in oxalate poisoning.

Citrates act in quite a different manner. Large doses do not reduce the concentration of blood calcium, but they do cause convulsions and death. Shelling and Maselow showed that the citrate unites with all of the calcium in the blood to form a citrate complex. The calcium is removed from combination with the proteins so that the total of the calcium becomes diffusible. Practically none of the calcium is left in its usual ionized state. Ammonium chloride and other acidosis producing compounds are said to produce a small increase in the concentration of serum calcium.

Morris, Rennie and Morris observed that intravenous injections of glycine in rabbits and goats reduced the calcium level by 1 or 2 mgm. per cent for a period of an hour. Intraperitoneal injections, however, were ineffective. The authors offer the explanation that glycine carries calcium into the tissue cells. The calcium is then released upon the deaminization of the glycine.

Involvement of calcium in the functioning of the sympathetic nervous system has received considerable attention in recent years. It has been pointed out by Zondek that the sympathetic system has an action similar to that which occurs when there is an artificial excess of calcium. In this worker's estimation, ions are the materials used by the nerves to fulfill their function, so that stimulation of the vagus produces a preponderance of potassium. Stimulation of the sympathetic system leads to a preponderance of calcium through the function of these nerves in controlling the distribution of these ions within cells and cell membranes. Leites claims to have experimentally verified this.

To test further this hypothesis, Berg, Hess, and Sherman carried out

experiments in which certain of the sympathetic nerves and the vagi were severed. Severance of various sympathetic nerves, according to these authors, led to a very marked drop in serum calcium to values as low as 6.0 mgm. per cent within 1 to 5 days. On the other hand, bilateral section of the abdominal vagi led to no significant alterations. On section of the spinal cord they state that a disturbance in the equilibrium of the blood calcium is induced, but the observed course is not uniform nor constant. Lamelas, on the other hand, was unable to confirm this work. He found that the calcium level remained normal in cats completely sympathectomized from the stellate to the pelvic ganglion or after section of the right or left splanchnic nerves. Neither did the nervous reactions produced by adrenalin injections or "sham rage" result in any noteworthy change in the amount of serum calcium. The inconsequential influence of adrenalin has also been reported by other workers.

A relation between the alkali earth elements and carbohydrate metabolism has from time to time been indicated. This apparent relationship has raised considerable interest in connection with views on the influence of certain agencies on the prolonged bleeding often associated with jaundice. After administration of insulin, glucose, or adrenalin, the amount of inorganic phosphorus of the blood and urine of normal subjects decreases, while that of the muscle and liver is increased. As the blood sugar level returns to normal, the level of the blood phosphate is restored. In severe experimental and clinical diabetes, the administration of glucose does not alter the phosphate concentration. The inorganic phosphate then is involved in the processes by which glucose is removed from the blood. Concurrently with the phosphate changes there is a small increase in the amount of serum calcium. Injection of serum calcium, it has been reported, conversely increases the blood sugar level. These changes have led to a controversy on the relation of calcium and glucose with respect to their beneficial effect in decreasing the bleeding tendency in jaundice. Both calcium and glucose do this. The subject of dispute is whether the calcium influence is due to a mobilization of glucose or vice versa.

Calcium therapy as a preoperative measure in jaundice has had a considerable popularity particularly through the efforts of Walters. In 1930 Ravdin indicated that preoperative glucose therapy has a most beneficial effect on controlling bleeding in jaundice. According to this worker, a good store of glycogen in the liver and a raised sugar level in the blood is of far more importance in increasing the coagulability of

the blood than is a raised calcium content. Wright and Cowan, however, have attributed the beneficial influence of glucose to its producing an increase in blood calcium. Working on jaundiced dogs they observed that, while a raised blood sugar is associated with an increased blood coagulability, the increased coagulability of the blood lags behind the sugar increase and persists for some hours even after the sugar has receded to normal. They further noted that glucose injection causes a rise in the amount of blood calcium and the increased coagulability seems to parallel the blood calcium curve more closely than it does that of the blood sugar. On the other hand, injection of hypertonic NaCl solutions, which leads to a moderate rise in blood sugar but not in calcium, caused no decrease in coagulation time. They reported increases in blood calcium by glucose injection of as much as 4.0 mgm. per cent which is far more than has been recorded by other workers. Rewbridge and Andrews, while admitting the beneficial effect of an increased blood calcium value on the clotting time, are, however, of the opinion that it is secondary to an influence on the carbohydrate metabolism. They ascertained that an injection of calcium induces a rise in the blood sugar level of nearly as great a magnitude as is produced by glucose itself. The average increase noted was around 70 per cent. Sterkin is of the opinion that calcium as well as potassium ions have no specific effect on the blood sugar picture although both induce a small degree of hyperglycemia.

Magnesium salts are said to be much more potent than calcium or other common electrolytes in causing an hyperglycemia and a glycosuria. While the glycosuria following magnesium sulfate injection was initially casually noted by Meltzer and Auer, the first careful study of its effect in this direction was carried out by Underhill and Closson. In the estimation of these workers, the hyperglycemic action of magnesium is the result of a disturbance in respiratory action. Lang and Rigo have carefully studied the action of magnesium salts on the blood sugar picture. These authors injected amounts of between about 25 to 100 mgm. of salt per kilo which amounts are much smaller than narcotizing doses. These doses increased the glucose concentration in the blood from 10 to 30 per cent. Lang and Rigo feel that they have ruled out the possibility of dyspnea or asphyxia as being in any way a factor in the action of magnesium on blood sugar. Instead, from the counteracting effect on the picture produced by autonomic drugs, they assume that the hyperglycemia is produced by an action of magnesium on the sympathetic nervous system.

The hypnotic and soporific action of magnesium and the counteracting effect of calcium upon it is a phenomenon of great interest and deserves attention. An extremely high plasma magnesium level is an important part of the picture. Neuwirth and Wallace state that a mild depression is produced in dogs when the serum magnesium level increases to over 5 mgm. per cent, and complete anesthetization is produced at a level of around 20 mgm. per cent. In their opinion, a similar relation holds for man, so that a serum magnesium concentration of 5 to 6 mgm. per cent is required to give a mild sedative action. Taylor and Winter determined 7 to 11 and 18 to 21 mgm. per cent respectively to be the levels for light and deep narcosis in the rabbit. They criticize the sedative level given by Neuwirth and Wallace because they observed that levels up to 10 mgm. per cent did not induce even light narcosis in the dog.

Very interesting is the observation of Taylor and Winter that magnesium lowers the temperature in fever, the observed therapeutic effect being related to the serum concentration rather than to the dosage, so that each increment of about 2 mgm. per cent in serum magnesium content causes a temperature reduction of about 1.0°F. Barbour and Taylor have observed a synergistic effect of magnesium upon barbital narcosis. They state that a mixture of 2 parts of magnesium chloride to 1 of sodium barbital hastens the onset and decreases the persistence of the narcosis without any significant increase in toxicity. According to Wokes, the narcotic dose of magnesium is only slightly lower than a lethal dose. Narcosis is not far removed from the terminal symptoms of the lethal effect of magnesium.

Meltzer and Auer also discovered the most interesting waking action of calcium salts upon magnesium anesthesia. The anesthetic limits of magnesium salts are between 0.1 and 0.2 gram per kilo when given subcutaneously. This dose varies somewhat in different animals. Higher doses than the upper limit are usually fatal. However, by giving calcium during the anesthesia, animals survive such large doses as 0.3 gram per kilo. With such a large dose a single injection of calcium ordinarily does not prove sufficient for permanent recovery. The first injection causes the anesthetized animal to awaken, but it will again sink into a comatose state. A second calcium injection usually completely re-awakens it. Meltzer and Auer proposed no theory for this relation of calcium and magnesium and nothing very satisfactory has since been proposed.

Mathews and Austin also state that a high calcium level reduces the toxicity of magnesium. The fatal dose of $MgSO_4.7H_2O$ for the dog

with a normal serum calcium level is between 0.23 and 0.28 gram per kilo. Dogs with hypercalcemia induced by parathyroid extract tolerate up to 0.3 gram per kilo. The pathological effect of the parathyroid injection on the heart and vascular system is somewhat ameliorated by injection of $MgSO_4$. Dogs with an hypocalcemia—as a result of parathyroidectomy or oxalate injection—are killed by doses of 0.14 to 0.19 gram of $MgSO_4.7H_2O$ per kilo.

The anesthetic action of magnesium and the waking action of calcium salts have been studied by Yamawaki. He points out that the narcosis is essentially due to the action on the central nervous system. Besides loss of consciousness this is attested to by the setting in of general anesthesia, the loss of reflexes, removal of the vagus inhibition of the heart, and in the terminal stages, a continuous fall in blood pressure and paralysis of respiration. The early loss of the standing reflex, and unconsciousness point to an involvement of the medulla oblongata. Yamawaki arrived at the opinion that the counteracting effect of calcium was also of a central nervous system origin. In other words, the waking action of calcium salts on magnesium narcosis, in this author's estimation, is not due to any fundamental physiological antagonism between calcium and magnesium, but rather to the differential action upon different parts of the brain. Calcium chloride produces irritability in the upper brain and in the corpus striatum. On the brain stem, calcium has a quieting action. In a thalamus animal calcium produces a quiescent and light sleep and thalamus rabbits, narcotized with magnesium, instead of being awakened by calcium, are more profoundly anesthetized. From this it is argued that the waking action of calcium probably depends upon the motor irritability of the corpus striatum.

One of the corner stones that forms the basis for linking of magnesium with calcium is the work of Mendel and Benedict on the path of excretion of magnesium introduced parenterally into the blood. In this study they showed that there was only a small excretion of magnesium sulfate into the intestines when injected subcutaneously into animals, the kidneys being predominantly important in the elimination of the excess magnesium. The injection of magnesium produces an increase in the excretion of calcium as well as of magnesium via the urine and conversely, injection of calcium salts is accompanied by a rise in the urinary output of magnesium.

Another strong proponent of the function of magnesium as a regulator of calcium metabolism is Bĕcka. In his estimation, magnesium regulates the biological relations of calcium in absorption, and also the

excretion from the organism. He estimates that a Mg:Ca ratio of 1:10 is required for this functioning.

4. BODY FLUIDS. *a. Lymph, transudates, synovial fluid and ocular fluid.* Special fluids, rather than the blood itself, bathe the different tissues of the body. These fluids are the media for the transport of the nutriments to, and the excretory products from, the tissues. The transport of the alkali earth elements and phosphorus between tissues and the organs of absorption and excretion must take place via these fluids as well as through the blood stream.

True interstitial fluid, it is believed, is formed by the dialysis or ultrafiltration of liquid from the blood plasma through the endothelial walls of the capillary bed. In certain organs of the body, the capillaries are specifically modified; for example, the capillaries of the kidney and of the choroid plexus of the central nervous system. In these tissues the capillary endothelium is reinforced by a second layer of either epithelial or endothelial cells. These membranes then are specialized structures and differ in function from the ordinary capillaries of most of the tissues of the body which only have a single layer of endothelial cells. It has as yet not been possible to obtain true interstitial fluid in amounts sufficient for analytical study.

For this reason, recourse is had to material which is assumed to be representative of interstitial fluid. In health the best representative is believed to be the lymph and, in certain diseases, such transudates as edema fluid, ascitic fluid, perivascular fluid, etc., offer a good measure of the composition of interstitial fluid.

The first concern is the nature of the mechanism which determines the composition of these different fluids. Until recently, it was generally assumed that normally only the capillaries of the liver and intestines were permeable to the plasma proteins, and that the capillaries of the rest of the body were assumed to permit the passage of only a protein-free dialysate. In inflammation or other abnormalities, however, the capillaries were assumed to become quite freely permeable to protein. A more modern conception, introduced by Drinker and Fields, is that the capillaries of all of the tissues are somewhat permeable to protein. The protein content of true interstitial fluid is assumed to be low, but becomes enriched by the time the fluid reaches the lymphatics through loss of water and salts to the blood stream. Normally there is no flow of protein from lymph or interstitial fluid back to the blood stream except by the lymphatic route. This has led to a theory that the capillaries are permeable to proteins in one direction only. Field

and Drinker have recently shown that this normal unidirectional exchange of protein is not due to a one way permeability, but instead is the result of the existing blood pressure and osmotic relations. If these are upset, and the blood pressure and the plasma protein concentration are temporarily lowered by plasmapheresis, protein as well as water and salts are then taken up directly by the blood.

Weech, Goetch, and Reeve have pointed out that the formation and composition of interstitial fluid may be explained according to two hypotheses. In the first it may be assumed that the capillaries are everywhere permeable to protein to about the same extent, the result of which is that the intercellular spaces become bathed in a fluid the protein content of which is a rough measure of the degree of capillary permeability. According to the second and more probable hypothesis, the capillaries contain pores of many different sizes, the greater number being too small to permit the passage of the plasma proteins. Of the larger pores some few will be of such a size as to hold back globulins, while the still larger ones will permit the passage of both. It should be added that much evidence exists which indicates that this size difference in the capillaries is functional, so that each capillary is capable of varying in size and permeability in accordance with physiological conditions.

The published data on the chemical composition of transudates and lymph are not plentiful. A summary of the figures obtained by Greene, Bollman, Keith, and Wakefield on the composition of ascitic fluid in dogs produced by ligation of the common bile duct and of ascitic or pleural fluid obtained from human subjects is given in table 10. This is the most extensive and complete investigation available. A careful study of thoracic duct lymph in the dog has been carried out by Arnold and Mendel. Some representative results from their data are given in table 11.

With an approximately normal composition of blood serum, the transudates contain a little less sodium, potassium, and phosphate, very much less calcium and magnesium, and more chloride and CO_2 than does the corresponding serum. The protein may vary from a few tenths of a per cent, as in the experimental ascites in the dog, to values not much less than is present in serum. In thoracic duct lymph the composition is more nearly akin to the blood serum, probably because of the usually higher protein content, but the features pointed out for transudates are still apparent. The work of Loeb, Atchley and Palmer, of Hastings and co-workers, and of Greene and associates, supports the opinion that the electrolytic composition of the transudates is the result

TABLE 10

The alkali earth elements and inorganic phosphate content of certain body fluids

	NUMBER OF OBSERVATIONS	RANGE*	MEAN	STANDARD DEVIATION	SOURCE OF DATA
(I) Transudates					
(a) Dog					
Calcium	10	6.4– 8.4	6.6	0.6	(1)
Magnesium	10	1.6– 2.8	2.1	0.2	(1)
Inorganic phosphate	10	3.5– 5.5	4.3	0.6	(1)
Protein, per cent	10	0 – 5.0	0.3	0.2	(1)
(b) Man					
Calcium	10	5 –10	7.5	1.0	(1)
Magnesium	10	1.8– 2.8	2.2	0.4	(1)
Inorganic phosphate	10	2.0– 5.0	3.5	0.7	(1)
Protein, per cent	10	1.0– 5.0	3.1	1.3	(1)
(II) Cerebrospinal fluid					
(a) Man					
Calcium	65	3.6– 6.4	5.3	0.6	(2, 3, 4, 5)
Magnesium	65	2.8– 4.0	3.3	0.2	(2, 3, 5)
Inorganic phosphate	48	1.0– 4.0	1.7	0.4	(2, 3, 4)
(b) Dog					
Normal					
Calcium	47	4.4– 8.0	5.8	0.6	(6, 7)
Parathyroid tetany					
Calcium	20	3.5– 6.5	5.0	1.3	(6, 7)
(III) Synovial fluid					
(a) Man					
Calcium			10.0		(8)
Protein, per cent		4.2– 7.3	5.2		(8)

* Analytical figures for inorganic substances are expressed in milligrams of element per 100 ml.

(1) Greene, C. H., J. L. Bollman, M. M. Keith and E. G. Wakefield. J. Biol. Chem. **91:** 203, 1931.

(2) Merritt, H. H. and W. Bauer. J. Biol. Chem. **90:** 233, 1931.

(3) Hamilton, B. J. Biol. Chem. **65:** 101, 1925.

(4) Fremont-Smith, F., M. E. Dailey, H. H. Merritt, M. P. Carroll and G. W. Thomas. Arch. Neurol. Psychiat. **25:** 1271, 1931.

(5) McCance, R. F. and E. Watchorn. Quart. J. Med. **24:** 377, 1931.

(6) Morgulis, S. and A. M. Perley. J. Biol. Chem. **88:** 169, 1930.

(7) Cameron, A. T. and V. H. K. Moorhouse. J. Biol. Chem. **63:** 687, 1925.

(8) Cajori, F. A., C. Y. Crouter and C. V. Pemberton. Arch. Int. Med. **37:** 92, 1926.

of a Donnan distribution between serum and fluid. Such a contention, while suggested, is not so clearly indicated for lymph. However, with certain modifying conditions to be kept in mind, an analogous distribution very probably obtains between serum and lymph. The ionic composition of true interstitial fluid, with hardly a doubt, is due to a rapid diffusion equilibrium with the blood plasma and so comes under the operation of a membrane distribution. This fluid, according to the references cited, has a much lower protein content than thoracic duct lymph. The suggestion is that as this fluid is taken up by the lymphatic system, water and diffusible constituents filter back into the blood stream so that the protein content becomes enriched. In the lymph

TABLE 11

A comparison of the composition of the serum and thoracic duct lymph in the dog

FLUID	CALCIUM	INORGANIC PHOSPHATE	PROTEIN
	mgm. per cent	*mgm. per cent of P*	*per cent*
Serum	10.4	4.3	5.6
Lymph	9.2	3.6	3.5
Parathyroidectomized			
Serum	7.0	5.1	6.3
Lymph	6.6		4.4
Injected with parathyroid extract			
Serum	17.3	6.9	4.8
Lymph	16.0	6.6	3.2

ARNOLD, R. M. AND L. B. MENDEL. J. Biol. Chem. **72:** 189, 1927.

ducts, then, the lymph may be expected to have become considerably modified from its initial composition and so is no longer in exact equilibrium with the blood plasma.

The analysis of the calcium, magnesium, and inorganic phosphate in the dog ascitic fluid, in which the protein content is very low, is of special interest. The amount of these substances present in this fluid is in good agreement with the content of diffusible calcium, diffusible magnesium, and diffusible inorganic phosphate found by ultrafiltration or vivi-dialysis. This agreement supports the conception of the simple dialysate nature of interstitial fluid. With increasing protein content in such fluids, of course, the amount of magnesium, and particularly of calcium, becomes increased and more nearly approaches

the content in the serum. The amount of inorganic phosphate present is not appreciably changed by an increase in protein content.

Another way in which the dialysate origin of these fluids is manifested is by their ready response to any alteration in blood composition. Arnold and Mendel found that there is a ready interchange between the blood and the lymph whenever fluctuations are induced in the diffusible constituents of the blood.

This was also found to hold true for glucose either when it was increased by injection or reduced by insulin. Similarly, when the serum calcium was reduced following parathyroidectomy, or increased either by calcium salts or by parathyroid extract, the changes in the blood picture were promptly mirrored in the lymph. Working on chest and edema fluids, Gilligan, Volk and Altschule found that there was an increase in the calcium and a decrease in the inorganic phosphate levels in fluids after the administration of parathyroid extract paralleling the changes of these constituents in the blood. From the observed changes, these authors concluded that parathyroid extract mainly increases the concentration of diffusible calcium.

Of the other body fluids, only synovial fluid appears also to be in ready diffusion equilibrium with the blood plasma. Cajori, Crouter and Pemberton have made an examination, by no means extensive, of synovial fluid. These authors point out that, aside from the knowledge that it plays a lubricating rôle in joint structures by virtue of its mucin content, little is known concerning the origin and functions of this fluid, particularly as to whether it serves as a nutrient medium. The protein content of this fluid (4.2 to 7.3 per cent) is nearly the same as is present in plasma.

These authors found about the same content of calcium in the synovial fluid as is present in blood serum, namely, 8.3 to 10.7 mgm. per cent. The content of the other diffusible constituents in synovial fluid is close to their concentration in the blood. Increases in blood sugar were observed to be promptly mirrored in the joint fluid. However, the presence of mucin and certain other constituents not found in the blood, shows that this fluid is somewhat more than merely a diffusate of the plasma.

The cerebrospinal fluid and the aqueous and vitreous humor of the eye have been claimed to be dialysates of the blood plasma. However, numerous difficulties in accepting this interpretation have appeared as the result of recent studies. Duke-Elder particularly has championed the view that the electrolytes of the aqueous and vitreous humor are in

Donnan equilibrium with the plasma. His data show a qualitative correspondence with this idea. In a careful study of ocular fluid, Stary and Winternitz found an average of 7.4 mgm. per cent of calcium contrasted with 12.1 mgm. per cent in the serum. Very interestingly, 7.4 mgm. per cent was also obtained for the ultrafiltrate value of this serum. From this it is apparent that the calcium content of ocular fluid, on the average, corresponds with the ultrafilterable fraction of serum calcium. Furthermore, the same authors found that dialysing ocular fluid against the serum produced only minor changes in the calcium concentration of both fluids. The average magnesium content of ocular fluid was found to be 1.7 mgm. per cent and, in the sera of the animals used, 1.8 mgm. per cent. The amount of ocular fluid magnesium, however, was quite a bit higher than was found in ultrafiltrates of the sera. Correspondingly, on dialysing ocular fluid against serum, the amount of magnesium in the fluid decreased. The distribution of the alkali elements, too, agrees fairly well with the requirements for a Donnan distribution.

Walker, in 80 experiments on a large variety of animals, found that the amount of inorganic phosphate of aqueous humor averages less than 50 per cent of the amount found in the plasma. The urea concentration in aqueous humor is only about 68 per cent of the amount present in the blood, while the concentration of reducing substances is only slightly less in ocular fluid than in the blood. Another difficulty in the way of the dialysate theory is the negligible response in ocular fluid to changes in blood composition. Merritt and Bauer found only a small change in the calcium content of the aqueous humor rabbits treated with parathyroid extract. While the amount of serum calcium increased on the average by 4.2 mgm. per cent, the average increase in the aqueous humor was only 0.6 mgm. per cent. Because of the discrepancies in composition for certain of the components, and the lack of a ready exchange between the eye fluids and the blood, the dialysate theory is difficult to accept.

b. Cerebrospinal fluid. In considering cerebrospinal fluid, one is confronted with an extremely thorny problem. The chemistry of this fluid has been very extensively studied, but highly divergent views still prevail regarding the nature of its origin. In an earlier period, physiologists attempted to decide the mechanism of its formation from the evidence of the histological changes in the epithelial cells of the choroid plexus and from the changes in pressure produced by the application of various secretagogues. The most common conclusion from these studies

was that cerebrospinal fluid is a true secretion. However, Fremont-Smith, on analysing this evidence, pointed out many discrepancies with the known behavior of true secretory glands, and from a variety of other evidence, arrived at the conclusion that the fluid is formed by ultrafiltration or dialysis from the blood plasma.

Since histological and physical evidences alone are insufficient to decide the problem, attention has been turned to the chemical composition for corroborative evidence.

Fremont-Smith and associates offer the close correspondence in the freezing point of cerebrospinal fluid and serum as evidence for the filtrate theory. However, it is pointed out by Walker, that gastric juice and hepatic bile, both true secretions, have freezing points nearly identical with that of blood serum. A comparison of the electrolyte composition of the fluid and the blood plasma, too, does not yield unequivocal evidence. Pincus and Kramer, the first to examine the subject, thought that the relationship might be explained by a Donnan membrane distribution. Several years following, Hamilton published a more accurate comparison on human subjects suffering from a variety of diseases. His decision was that "although indications are not lacking that an equilibrium of the Donnan type may, at least partly, govern the distribution of electrolytes between serum and spinal fluid, it seems probable that the equilibrium is modified by unknown factors."

Greenberg has pointed out that, even if it is accepted that the cerebrospinal fluid is a dialysate of the blood plasma, there still remains the question of whether it is continuously in equilibrium with and gives a true picture of the changing states of the diffusible constituents in the plasma, or if it is only rarely in actual diffusion equilibrium with the blood plasma. The anatomical relations involved in the exchange between fluid and plasma certainly do not appear to favor rapid diffusion.

The figures for chloride and calcium and the greater alkalinity of spinal fluid are in qualitative agreement with the demands of a Donnan distribution. The distribution of the rest of the electrolytes is difficult to fit into this picture. The work of Fremont-Smith and associates, which shows that the cerebrospinal fluid contains slightly more sodium than the plasma (average ratio 1.02), and much less inorganic phosphate (average ratio 0.39), does not fit in with the requirements of a membrane distribution. Similarly, Bario, Cohen, and Watchorn and McCance have thoroughly established that, contrary to the dialysis theory, the magnesium of the cerebrospinal fluid is on the average about 3.3 mgm. per cent, while the plasma contains about 2.5 mgm. per cent. The

recent analyses of Watchorn and McCance on the potassium distribution between cerebrospinal fluid and blood plasma, too, run counter to the dialysate theory.

Updegraff, Greenberg and Clark have pointed out that an examination of the comparative distribution of non-electrolytes between the blood and cerebrospinal fluid is of interest in connection with the problem of the nature of the cerebrospinal fluid, because these substances are free from the complexities of the Donnan membrane distribution. Careful studies of this distribution have recently been published by Cockrill and by Fremont-Smith and co-workers. The consensus of this work is that the level of nearly every non-electrolyte is a good deal lower in the spinal fluid than in the blood plasma. Cockrill obtained 17 to 67 per cent as much uric acid, 43 to 98 per cent as much creatinine, 21 to 82 per cent as much glucose, and 20 to 66 per cent as much urea as was present in the serum of the same animal. In nephritic cats, the urea content, it was found, increased in both plasma and the cerebrospinal fluid, but the ratio between the two fluids remained nearly unchanged. On the other hand, when plasma was dialyzed against the simultaneously obtained cerebrospinal fluid, the urea in the fluid increased to a level slightly higher than its content in the plasma. The work on non-electrolytes, then, would appear to be even more opposed to the theory of a dialysate formation than are the anomalies in electrolyte distribution.

In the light of the above, it is curious that the calcium content of cerebrospinal fluid is often in close agreement with the amount of diffusible calcium of the serum as determined by ultrafiltration. Greenberg pointed this out in a small series of cases. It has been corroborated by Searle and Michaels from an examination of some 80 inmates of the State Psychopathic Hospital of Michigan. These authors found all of their cerebrospinal fluid calcium analyses to be between 84 and 135 per cent of the respective ultrafiltrate values, with 70 per cent of the cases falling between 90 and 110 per cent. In the estimation of these workers, the frequent close agreement between cerebrospinal fluid and serum ultrafiltrate calcium in their series can scarcely be dismissed as merely fortuitous. No comparable directly measured series of analyses have been carried out on the dog. The cerebrospinal fluid calcium was found by Morgulis and Perley, and by Cameron and Moorhouse, in untreated dogs (see table 10), to vary between 4.4 and 8.0 mgm. per cent, with a mean of 5.8. This figure is more than 90 per cent of the mean diffusible calcium found in the dog either by the ultrafiltration or the vivi-dialysis method.

This correspondence, however, is maintained only while the level of serum calcium remains about normal. Cameron and Moorhouse and Morgulis and Perley observed only a slow and small reduction in the amount of cerebrospinal fluid calcium of parathyroidectomized dogs whose level of serum calcium was greatly lowered. Conversely, Morgulis and Perley and Merrit and Bauer found that the spinal fluid calcium shows only a small increase when the concentration of blood calcium is increased with parathyroid extract.

A number of investigators who have assumed that the cerebrospinal fluid is a protein free filtrate of the plasma have been led astray in interpreting the mechanisms involved in tetany and other conditions in which calcium plays a rôle. The belief in the dialysate nature of the cerebrospinal fluid appears to be widespread in certain clinical circles, and on this assumption, analyses of the calcium in the fluid have been used to estimate the diffusible or physiologically active calcium for diagnostic purposes. This is unfortunate since, as has just been pointed out, the cerebrospinal fluid calcium practically does not reflect any marked alterations of this element in the blood stream.

5. CALCIUM, MAGNESIUM AND PHOSPHORUS IN TISSUES AND SKELETON.

a. Tissues. A summary of representative data on the calcium, magnesium, and phosphorus content of certain of the organs and tissues of the animal body has been collected in table 12. Some of the data in this table are given on a moist weight and others on a dry weight basis. The water content of most of the soft tissues varies between 75 and 80 per cent. The dry weight analysis then will be some four to fivefold as great as when referred to the moist weight. As table 12 shows, reliable data of this kind are indeed extremely meagre. One also is struck by the great variability in composition of the tissues in contrast to the quite constant figures for these elements, found in blood, other body fluids, and in bone.

Denis and Corley have pointed out that the inconstancy is partly due to the difficulty of obtaining a true sample of a tissue because of the fluctuating amount of interstitial fat, of blood, and of connective tissue present. Partly also, the observed variability may be due to analytical defects, since even now the methods of analysis for tissues are still mostly in a crude state. In spite of the drawbacks pointed out, it is still probable that there is a considerable degree of inconstancy in the composition of the soft tissues. This makes it difficult to decide whether differences observed with a given type of experimentation are actually a reflection of that procedure. A good many contradictory statements existing in the literature have resulted from this uncertainty.

Table 12 shows that magnesium is essentially a tissue or cellular cation in contrast to calcium, which preponderates in the body fluids. Phosphorus, of course, is present in enormous amounts in the tissues compared with the two cations, namely, 10 to 20-fold that of the calcium or magnesium content.

The chemical states in which the calcium and magnesium occur in the soft tissues are only slightly known. Quagliariello and Sjollema and Seekles have determined the composition of muscle fluid obtained by subjecting muscle tissue to 25 and 200 atmospheres' pressure. The results of the latter authors which appear to be the more reliable are about 10.0 mgm. per cent of calcium and 20.0 mgm. per cent of magnesium. Muscle fluid obtained in this way contains considerable amounts of protein. On being subjected to ultrafiltration, the diffusible calcium and magnesium of the muscle fluid were found to be 8.0 and 15.0 mgm. per cent respectively.

A small amount of magnesium, according to Lohmann, is combined with the adenylpyrophosphate present in the muscle. Magnesium also is known to be necessary for the activity of the enzyme phosphatase. Little else is known about the exact functions of the two cations.

We shall now examine a few of the tissues separately. Determinations on human brain show an average calcium content of 4.5 mgm., 10.0 mgm. of magnesium, and 160 mgm. of total phosphorus per 100 grams of moist brain. Since most tissues contain 75 to 80 per cent of water, the dry weight content of these elements should be 4 to 5 times the figures for the moist weight. In agreement with this the average calcium content on a dry weight basis has been reported as 20 mgm. per cent in the human, and 20.6 mgm. per cent in the dog. From these results, the calcium analyses of rat brain on a dry weight basis (mean 183 mgm. per cent), reported by Hess and co-workers, seem astonishingly high. According to these workers, rickets produces a considerable reduction in the calcium, but none in the phosphorus content of rat brain. No reduction in either quantity was found in tetany. The same authors state that they could lower the amount of brain calcium by diets low in calcium and high in phosphorus. The brains of rachitic rats treated with cod liver oil or viosterol in amounts sufficient to induce healing, it was found, did not at once increase in calcium. However, after complete healing, the amount of calcium did increase slowly toward normal. No change has been observed in the content of calcium in the brain in other diseases.

TABLE 12

Calcium, magnesium and phosphorus content of body tissues

ANIMAL	CONDITION	BASIS OF ANALYSIS	CALCIUM		SOURCE OF DATA	MAGNESIUM		SOURCE OF DATA	PHOSPHORUS		SOURCE OF DATA
			Range	Mean		Range	Mean		Range	Mean	
Brain											
			mgm. per 100 gm. tissue			*mgm. per 100 gm. tissue*			*mgm. per 100 gm. tissue*		
Man	Normal	Wet weight	3.2– 5.8	4.5	(1)	6 –14	10.0	(2)	140– 180	169	(1)
Dog	Normal	Dry weight	24 – 202	55.4 ±14	(3)				1125–1526	1317 ±31.5	(3)
Dog	Viosterolized	Dry weight	34 – 260	125 ±16.7	(3)				312–1968	1018 ±130	(3)
Rat	Normal	Dry weight	100 – 264	183	(4)				973–1468	1278	(4)
Rat	Rachitic	Dry weight	36.9– 77.5	59	(4)				1104–1451	1314	(4)
Rat	Parathyroidectomized	Dry weight	101 – 343	—	(4)				1100–1400	—	(4)
Heart											
Man:											
Right ventricle	Normal	Wet weight	5.9– 9.3	7.6	(5)	16.4–22.3	19.8	(5)	142– 203	177	(5)
Right ventricle	Normal	Dry weight		36.9	(5)		95.4	(5)		852	(5)
Left ventricle	Normal	Wet weight	6.0– 9.5	6.9	(5)	15.6–23.2	20.1	(5)	179– 244	203	(5)
Left ventricle	Normal	Dry weight		32.7	(5)		95.0	(5)		962	(5)
Right ventricle	Cardiac disease	Wet weight	5.4– 9.9	7.1	(5)	11.6–18.5	14.7	(5)	122– 183	149	(5)
Left ventricle	Cardiac disease	Wet weight	4.4– 10.0	6.2	(5)	14.0–18.0	15.8	(5)	142– 188	170	(5)
Dog:											
Right ventricle	Normal	Dry weight	13 – 87	27.7 ±4.4	(3)				581–1008	708 ±38	(3)
Left ventricle	Normal	Dry weight	12 – 38.4	18.3 ±1.6	(3)				513–1094	767 ±50	(3)
Right ventricle	Viosterolized	Dry weight	17 – 517	150 ±38	(3)				201–1009	695 ±49	(3)
Left ventricle	Viosterolized	Dry weight	17 – 969	220 ±64	(3)				409–1705	994 ±127	(3)
Bird	Normal	Wet weight		5.4	(7)		12.5	(7)		140	(7)
Kidney											
Man	Normal	Wet weight		19	(8)		21.5	(8)	127– 192	168	(6)
Dog	Normal	Wet weight		41.3	(9)		12.6	(2)			
Dog	Normal	Dry weight	29 – 177	55.6 ±16	(3)				354–1112	816 ±74	(3)
Dog	Viosterolized	Dry weight	47 – 3460	672 ±236	(3)				409–1705	994 ±127	(3)

Liver											
Man	Normal	Wet weight		7.2	(8)		17.5	(8)	184– 305	241	(6)
Beef	Normal	Wet weight	8.0– 8.2	8.1	(9)						
Dog	Normal	Dry weight	10 – 28	18 ±1.2	(3)				512– 990	790 ±24	(3)
Dog	Viosterolized	Dry weight	20 – 678	123 ±45	(3)				351–1410	682 ±104	(3)
Lungs											
Dog	Normal	Dry weight	42 – 221	89 ±13	(3)				555–1143	865 ±53	(3)
Dog	Viosterolized	Dry weight	61 – 1860	338 ±160	(3)				509–2440	978 ±164	(3)
Muscle											
Man	Normal	Wet weight	3.6– 13.7	7.6 ±2.5	(6)	12 – 23.7	18.4 ±2.9	(6)	100–200	150 ±27	(6)
Dog	Normal	Wet weight	3.5– 7.0	4.9	(10)		27	(2)			
Dog	Normal	Dry weight	12 – 59	17.8 ±1.2	(3)				409–940	699 ±44	(3)
Dog	Viosterolized	Dry weight	14 – 227	65 ±13	(3)				151–784	537 ±48	(3)
Cat	Normal	Wet weight	4.3– 6.1	5.1	(10)						
Rabbit	Normal	Wet weight	4.1– 8.3	5.8	(10)						
Rat	Normal	Wet weight	5.2– 8.7	6.5 ±1.4	(10)						
Bird	Normal	Wet weight	1.3– 17	9.1	(7)	4.5– 9.4	6.9	(7)		230	(7)
Spleen											
Man	Normal	Wet weight		9.5	(8)		14	(8)			
Dog	Normal	Dry weight	7 – 32	14.3 ±2.2	(3)				682–1200	925 ±41	(3)
Cat	Normal	Wet weight	7 – 10	8	(11)						
Skin											
Man	Normal	Dry weight	30 – 60	46.5	(12)	20 – 40	30	(12)			
Dog	Normal	Dry weight	30 – 60	42.5	(12)	20 – 40	22	(12)	73– 344	152 ±37	(12)
Rabbit	Normal	Dry weight	50 – 86	74	(12)	20 – 55	37	(12)			
Rat	Normal	Dry weight	23 – 42	33	(12)	39 –555	47	(12)	405– 812	457	(12)
Bird	Normal	Dry weight		130	(7)		36	(7)		160	(7)

(1) Eaves, C. Brit. J. Exp. Path. **12**: 112, 1931.
(2) Javillier, M. M. Bull. Soc. Chim. Biol. **12**: 709, 1930.
(3) Reed, C. I., L. M. Dillman, E. A. Thacker and R. J. Klein. J. Nutr. **6**: 371, 1933.
(4) Hess, A. F., J. Gross, M. Weinstock and F. Berliner. J. Biol. Chem. **98**: 625, 1932.
(5) Wilkins, W. E. and G. E. Cullen. J. Clin. Invest. **12**: 1063, 1934.
(6) Cullen, G. E., W. E. Wilkins and T. R. Harrison. J. Biol. Chem. **102**: 415, 1933.
(7) Bernardi, A. and M. A. S. Schwarz. Biochem. Ztschr. **262**: 387, 1933.
(8) Magnus, L. A. Biochem. Ztschr. **24**: 363, 1910.
(9) Denis, W. and R. C. Corley. J. Biol. Chem. **66**: 609, 1925.
(10) Burns, C. M. Biochem. J. **27**: 22, 1933.
(11) Heubner, W. and P. Rona. Biochem. Ztschr. **135**: 248, 1923.
(12) Brown, H. J. Biol. Chem. **68**: 729, 1926.

Reed, Dillman, Thacker and Klein found that toxic doses of irradiated ergosterol fed to dogs more than doubled the calcium content of the brain, but produced practically no change in the amount of phosphorus. An enormous increase in calcium and little change in the phosphorus was found in every soft tissue of the body of ergosterolized dogs by these authors. On the other hand, Heubner and Rona could detect no change in the calcium content of the brain of cats injected with massive doses of calcium salts. Similarly, Stransky found no change in the calcium and magnesium content of the brain in narcosis induced by magnesium sulfate injection. Isolated brain tissue bathed in solutions containing magnesium, however, did show some permeability to the magnesium.

The composition of the heart in human subjects has recently been extensively studied by Wilkins and Cullen, and by Cullen, Wilkins and Harrison. A summary of their results is collected in table 12. These authors feel that some significance may be attached to the average differences found between the calcium, magnesium, and phosphorus composition of the right and left ventricles. Since a variability of nearly 100 per cent was found in the composition, this seems doubtful. It is to be noted that the average composition of heart for these elements does not differ greatly from that of brain or striated muscle.

Disease seems to influence the content of these elements in the heart very little, since the difference in the analyses between normal hearts and the hearts of subjects suffering from myocardial lesions, or a miscellaneous number of other diseases, is neither constant nor striking.

A good deal of work has been carried out on the calcium content of muscle by Burns, Denis and Corley, Heubner and Rona, and others; but little, however, is available on the amount of magnesium and phosphorus in this tissue. The content of these elements in muscle approximates their amount in the heart and other soft tissues. Mammalian muscle appears to be of the same composition in all species. Table 12 illustrates this for man, the dog, the cat, the rabbit and the rat.

No marked deviation from the average calcium content of muscle has been observed under abnormal circumstances except in overdosage with irradiated ergosterol. It is true that claims for such variations have been made. Haury states that there is 40 per cent less calcium in the muscles of rachitic rats than there is in normal rat muscle. Bulbring claims to have produced differences in the amount of muscle calcium by varying the calcium content of the diet. The majority of investigators, however, do not corroborate these authors. Burns observed no signifi-

cant changes in the calcium content of the muscles of rats following parathyroidectomy, dosage with irradiated ergosterol, injection of guanidine or dosage with phosphate solution. Dixon and Davenport detected no change in the content of calcium, the acid soluble phosphorus, nor in the partition of other phosphorus compounds in the muscles of dogs with tetanic convulsions following parathyroidectomy. Neither could Denis and Corley demonstrate any alterations in the amount of muscle calcium of rabbits dosed with calcium salts, nor could Heubner and Rona find any differences in the content of calcium in the muscle of cats similarly treated. No alterations in the magnesium content of the muscle were noted by Stransky in animals narcotized with magnesium sulfate. The significance of these findings to theories of the causation of tetany is discussed in another section.

The data on other vital organs are too scanty for lengthy discussion. Some figures on the analysis of the kidney, liver, lungs, and spleen are given in table 12. Apparently the lungs, kidney and intestines have a higher calcium content than do most of the other organs. In the work of Heubner and Rona, the kidney was the only tissue which significantly increased in calcium content after dosage with calcium salts. This organ appears to be especially susceptible to calcium deposition and enormous increases have been noted following the administration of irradiated ergosterol. Similar increases probably take place after overdosage with parathyroid extract.

Extensive studies have been carried out on the chemical composition of the skin of many animals. On a dry weight basis there is considerably more calcium and considerably less magnesium and phosphorus in the skin than there is in most of the other soft tissues. The work of Brown indicates that there is a very sharp increase in the calcium and magnesium content of the skin in fetal life which lasts up to a short time following birth. Thereafter these elements undergo a slow continuous reduction in amount during the rest of the life period. This author also points out that rabbit skin contains the greatest, that of man less, and that of the dog the least amount of calcium and magnesium. The sodium and potassium content follow quite the opposite variation. Brown is of the opinion that this alteration in composition is due to the differences in diet of these animal species.

The most striking deductions to be drawn from the analyses of the soft tissues is their rather large variability in normal composition and the slight influence which is exerted by most diseases or experimental procedures on the content of calcium, magnesium and phosphorus.

b. The skeleton. The solid supporting structure of the skeleton is a mineral material composed largely of carbonates and phosphates of calcium and magnesium. About one-third of the bone matrix consists of a protein substance, ossein or collagen. The remaining two-thirds are composed of the inorganic material mentioned above with a small admixture of the alkali elements. While the histological changes involved in bone mineralization are well known, the physical chemical processes which result in the laying down of the inorganic matrix are in a singularly obscure state.

The inorganic matter of fresh bone continuously increases during growth up to adolescence. The composition of the bone ash, however, shows only little change. A few representative figures of the elementary composition of normal bone ash are given in table 13. As the table

TABLE 13

Composition of bone

ANIMAL	CONDITION	CALCIUM	MAGNESIUM	PHOSPHORUS	CO_2	SOURCE OF DATA
		In per cent of total bone ash				
Dog	Normal	35.7	0.46	15.8	5.6	(1)
Cow	Normal	36.1	0.74	16.4	4.6	(1)
Rabbit	Normal	36.3	0.53	16.0	5.7	(1)
Hen	Normal	37.2	0.51	16.4	5.5	(1)
Rat	Normal	37.5	0.85	18.5	—	(2)

(1) MORGULIS, S. J. Biol. Chem. **93**: 455, 1931.
(2) HAMMET, F. S. J. Biol. Chem. **64**: 693, 1926.

shows, the ash of bone contains about 36 per cent calcium, 0.5 per cent magnesium, 16 per cent phosphorus, and 5.5 per cent CO_2. From chemical considerations, one would expect that the mineral matter of bone probably consists of the slightly soluble salts, $Ca_3(PO_4)_2$, $CaCO_3$, and $Mg_3(PO_4)_2$. Assuming this to be so, it has been calculated by Morgulis that bone ash is composed of about 80 per cent $Ca_3(PO_4)_2$, 13 per cent $CaCO_3$, 2 per cent $Mg_3(PO_4)_2$, and a small undefined residue of an unknown calcium salt and alkali salts. In the analysis by this author, the residual ratio of calcium to phosphorus after the amount of calcium equivalent to the CO_2 has been subtracted, varied between 1.99 to 2.12. This indicates that there is a greater amount of calcium present in bone than can be accounted for if it were merely tertiary phosphate and carbonate. The inorganic composition of bone is roughly, but by

no means strictly, constant. Pathological calcifications too have the approximate composition of normal bone. Howland, Marriott and Kramer obtained closely the same analytical figures among the different bones of the skeleton of a single individual, and Bogert and Hastings observed no material difference in the inorganic composition of the inner and outer portions of bone. On the other hand, Morgulis has shown that the mineral composition of bone varies considerably among different animal species. Differences in composition are observed at the various stages of development of the mammalian organism. Variations are found in pathological conditions. Howland, Marriott and Kramer noted a lower ratio of calcium phosphate to calcium carbonate in rachitic than in normal rats. The bones of subjects with rickets and also with osteomalacia, it has been reported, contain greater than normal amounts of magnesium. The answer to this may perhaps lie in the greater proportion of organic matter which is present in bone in these diseases.

Until recently, it has been the general opinion that the mineral matter in bone consisted merely of a mixture of the phosphates and carbonates of calcium and magnesium. Recent work, particularly of the x-ray spectrogram of bone, has popularized a much earlier viewpoint of Werner and others, that a complex mineral salt may actually be the main constituent of bone. All investigators in this field are agreed that it has a crystalline structure. Similarly, it is agreed that there is no evidence for the independent existence of $CaCO_3$ or $CaHPO_4$ as such in bone. The possible rôle of the latter salt in the physiology of bone deposition has been particularly emphasized by Kramer and Shear. In its crystalline form, it would be identical with the mineral brushtite. As another piece of evidence against the presence of $CaHPO_4$ in bone, it has been pointed out by Morgulis that on incineration this compound would be converted to pyrophosphate. This has not been found in incinerated bone.

The further general conclusion which has been drawn from the x-ray work is that the bone salt belongs to the apatite series of minerals. Beyond this there is no general agreement. Taylor and Sheard favor podolite, $CaCO_3 \cdot 3Ca_3(PO_4)_2$, as the chief mineral substance; Roseberry, Hastings and Morse have suggested the composition, $CaCO_3 \cdot nCa_3(PO_4)_2$, in which n is not less than 2 nor greater than 3. From chemical evidence, Klement and also Morgulis believe that the compound, $Ca(OH)_2 \cdot 6Ca_3(PO_4)_2$, is the most probable bone substance. From what has been said of the fluctuations in the inorganic composition of bone, it is clear that no single compound can account for the mineral matter in bone.

Even if in the main it is composed of a single complex salt, it is probably admixed with other slightly soluble salts. Due to the lability of the mineral matter in bones, it is not surprising that the composition of bone is not entirely constant.

The problem of bone calcification has been attacked by many investigators. Explicitly or tacitly, all theories of calcification assume as an important step in the mineralization of bone, the production of a supersaturated condition of one or more calcium salts, and the subsequent incorporation of the precipitated solid salt in the organic matrix. To obtain an insight into this phase, considerable work has been carried out on the solubility of certain slightly soluble salts which might be deemed to be solid phases in this process, namely, $Ca_3(PO_4)_2$, $CaCO_3$, and $CaHPO_4$. The work of Holt, La Mer and Chown indicates that all body fluids are normally supersaturated with respect to $Ca_3(PO_4)_2$. In their estimation, precipitation of this salt is determined by the degree of supersaturation and the slow reaction rate involved. The objections that have been raised to this particular salt as constituting a solid phase are: a crystalline salt of the above composition has never been demonstrated. When $Ca_3(PO_4)_2$ is equilibrated with biological fluids, $CaCO_3$, not $Ca_3(PO_4)_2$, is removed from solution. It has been determined that the ion product of both $CaCO_3$ and $CaHPO_4$ are near their theoretical saturation levels in blood plasma and other biological fluids.

The weakness of this work is the uncertainty of the relation of these salts to the solid phase actually present in bone. To define solubility exactly, it is obvious that the nature of the solid phase should definitely be known; otherwise, the critical ion product necessary for deposition cannot be precisely stated. Also, as has been pointed out by Taylor and Sheard, the ionic equilibria may vary in an incomprehensible way if unnoticed changes take place in the nature of the solid phase.

However, if the precipitation of one or more than one of the above mentioned salts is an initial or intermediate step in the process of the final formation of the solid substance of bone, then a knowledge of their solubility relationships becomes important in characterizing the reaction involved. A great deal of further information is still required to clarify the physical chemical aspects of bone deposition.

Besides the physical factors which determine solubility, without a doubt, very definite biological factors are involved in normal and pathological calcifications. Cartilage, it has long been known, has a selective action particularly favorable to calcification. As an illustration of this property, Wells showed that cartilage implanted in the abdominal cavity

takes up a large amount of calcium and phosphate in a ratio approximating the composition of the mineral in bone. Another example of a biological factor appears in the work of Shipley, Howland and Kramer on "in vitro" calcification. These authors noted that while the product of calcium and phosphorus was a determining factor for calcification, it was also essential to have living cartilage present. Also they observed a cessation of calcification upon the introduction of such protoplasmic poisons as HCN. Among the biological factors, there has been suggested by Robison the importance as an intermediary of an enzyme phosphatase which is quite prevalent in bone. The rôle of this enzyme, it is assumed, is to hydrolyse organic esters of phosphoric acid, and so by increasing the inorganic phosphate ion concentration, enable a readier extrusion of a slightly soluble salt of calcium and phosphate. It is to be noted that the action of phosphatase in calcification implicitly postulates the necessity of exceeding the saturation limit of some slightly soluble calcium salt. The weakness of the theory of phosphatase as an intermediate in bone formation is that the plasma which must furnish the substrate for phosphatase action contains only a trifling amount of the required phosphorus acid esters; equivalent to only about 0.5 mgm. per cent of phosphorus. As with physical chemical aspects, there exists only fragmentary knowledge of the biological mechanisms of calcification.

6. VITAMINS. *a. Rickets.* Of the vitamins, only D has a strongly marked influence on the elements considered in this review. The outstanding illustrations of the effect of a deficiency in vitamin D are rickets in the young animal, and osteomalacia in the adult. The conspicuous change in the blood picture which is found in rickets is a lowered percentage of inorganic phosphate. Ordinarily, the calcium level is nearly normal or only slightly lowered. Illustrative figures on the blood composition in rickets and other diseases in which the alkali earth elements and phosphorus are involved are given in table 9. The low blood phosphorus level in rickets appears to have been first pointed out by Howland and Kramer. These authors further made the generalization that the product of calcium and of inorganic phosphorus in the serum expressed in milligram per cent, gives an index of the severity of the disease and also of the degree of healing that may be anticipated from curative agencies. According to this scale, severe rickets is found when the Ca $\times$ P product is 30 or less. Healing may be anticipated when the product rises to over 40, and rapid healing when it is between 50 and 60. In the eyes of Hess, Weinstock, Rivkin and Cross, the calcium phosphorus product, while it may be valuable, is not an infal-

lible criterion of rickets in infants and even less so in experimental rickets. With rats these authors were able to induce cases of rickets during which the serum inorganic phosphate remained at 6.5 or 7 mgm. per cent by the addition of a small amount of milk or milk powder to the usual rachitogenic ration. They also demonstrated that in diets with the extremely high calcium to phosphorus ratio of 11.5 to 1, there was no healing produced by cod liver oil or irradiated ergosterol, even though the calcium and phosphorus product was favorable. The interpretation of these authors is that the healing of rickets is not merely contingent on the phosphorus level of the blood, but also upon an additional local factor. However, it is attested to by the cumulative experience of many years that the Ca × P product is ordinarily a useful guide to the healing of rickets. Usually, this product is raised by the administration of vitamin D or when the formation of this vitamin is induced by irradiation. The blood phosphorus level then returns to normal. The first action of this vitamin would appear to be on the content of inorganic phosphorus. We shall have occasion to refer shortly to quite a different action when vitamin D is administered in large doses.

Healing of rickets often may be brought about by other means than the administration of vitamin D. It can start spontaneously as a result of starvation, as has been observed by Cavins and Wilder. In such an event, the animals very often go into tetany. Analysis shows that the amount of serum calcium is reduced and the inorganic phosphate level is increased in these animals. Figures of serum calcium as low as 5.4 and serum phosphorus as high as 16 mgm. per cent have been reported. It would seem that the increased inorganic phosphate during starvation comes from the increased tissue catabolism. A destruction of body protoplasm in amounts which are sufficient to account for the observed rise in phosphorus is indicated by the change in urinary nitrogen.

Analogous induction of tetany has been brought about by diets very favorable to healing by Shohl and Bennett and by Hess, Weinstock, Benjamin and Gross. The latter authors produced this effect by changing the ration of rachitic animals to one of dried milk or milk and whole wheat. On these diets, a fall in serum calcium and an increase in serum inorganic phosphate concentration is produced. The reduced calcium level is maintained for only a few days after which it returns to normal. The induction of tetany by these means is highly important in relation to infantile tetany, which too, invariably comes about as a sequel to rickets.

Stare and Elvehjem have studied the change in other forms of phos-

phorus besides inorganic, in rachitic calves. They found only a small reduction in the amount of lecithin and other phosphorus esters. Only the level of inorganic phosphorus, it would appear, is strikingly altered. Magnesium is ordinarily not considered in connection with rickets, and probably rightly so. Howland and Kramer found that the amount of magnesium averaged 2.2 mgm. per cent in the serum of rachitic infants. Their normal values in infants averaged 2.6 mgm. per cent. Bomskov and Kruger state that the level of serum magnesium is lowered about 50 per cent in severe rickets and in rickets produced by thallium.

A relation between the functioning of vitamin D and the parathyroid hormone has been suggested from the work on rickets because hyperplasia of the parathyroids has been observed in both human subjects and in the experimental forms of this disease in animals.

b. Osteomalacia. This disease and certain related disorders are similar in their pathogenesis to rickets. Osteomalacia is said to be extremely prevalent in China and in India. The cause, it appears, is that the diet is low in calcium, high in phosphorus, and deficient in vitamin D. This is accentuated during pregnancy and lactation by the extreme demands upon the maternal organism. There is a softening of the bones, chiefly affecting the endosteal and periosteal calcification of the bones of the pelvis and back. Since the disease usually appears following closure of the epiphyses, the histological picture differs widely from rickets. Blood chemical studies generally show a low content of calcium and inorganic phosphate. Barr quotes from European sources average values of 6.0 mgm. per cent for calcium and 2.7 for inorganic phosphate. As might be expected from the low calcium level, tetany is almost invariably an accompaniment of the disease. Miles and Feng report similar analytical values in Chinese women. Calcium therapy and cod liver oil relieve the condition according to these authors. This finding is confirmed by Hughes, Shrivastava and Sahai. Following improvement, the amounts of both calcium and phosphate are markedly increased.

c. Excess vitamin D. The effect of vitamin D on the blood does not terminate with the increase in the amount of inorganic phosphate observed during the healing of rickets. In non-rachitic animals sufficiently large doses produce a small increase in the phosphate content, but a much more striking increase in the amount of serum calcium. The usual means of offering large doses of vitamin D is through the use of irradiated ergosterol. Vitamin D given in the form of cod liver oil or formed in the animal itself by exposure to irradiation, evidently has

never reached a sufficiently high dosage to produce the augmentation noted with irradiated ergosterol, since the experiments through these agencies are negative. Harris and Moore state that rats on a diet with 15 per cent of cod liver oil have a lower growth rate than normal. Failure of pregnancy also takes place in these animals.

Hess, Weinstock and Rivken found that 1 mgm. daily of irradiated ergosterol increased the low serum calcium level of rats raised on a diet high in phosphorus but almost devoid of calcium to nearly normal values. From this it can be inferred that the effect may be directly on the serum calcium. It is now almost universally conceded that the extra calcium poured into the blood stream under these conditions comes from a demineralization of the bones. According to Jones and Robson, the femurs of rats on high doses of irradiated ergosterol show very marked degenerative changes. The bones become very porous due to the removal of organic as well as inorganic matrix. The bones became very soft and fragile. Chemically, they showed a relative decrease in the ash content, apparently due to the subsequent growth of osteoid tissue following the absorption of the bone matrix. Histological studies indicate the bone destruction is due to osteoclastic activity.

Among the gross symptoms of overdosage with irradiated ergosterol are rapid loss of weight and anorexia. Harris and Moore state that atrophic changes in the spleen and thymus are found. Heavy calcium deposits form in the blood vessels, heart musculature, kidneys, lungs, and other tissues. Urinary calculi may be present. Comparison of the tissues of 13 normal dogs with those of 14 dogs dosed with irradiated ergosterol by Reed, Dillman, Thacker and Klein showed enormous increases in calcium content. Some of the figures are listed in table 12. On the other hand, the amount of inorganic serum phosphorus increased only slightly. The magnitude of the increase in serum calcium, however, could not be correlated with the dosage of irradiated ergosterol. Reed and associates feel that the calcification depends on other undetermined factors as well as on the irradiated ergosterol. The pathological lesions often also include hemorrhage of the intestines which may also involve the stomach. Ulceration is a frequent accompaniment according to Hess et al.

The cause of the calcium deposition has occupied some attention. Reed, Dillman and Thacker are of the impression that the cellular injury occurs first and the calcium deposition is probably a direct result of such an injury. On the other hand, they state that injury can often not be demonstrated histologically even though calcium deposition

takes place. Ham, who has studied the effects following the administration of huge single doses of irradiated ergosterol, pointed out that the striking feature is the extreme rapidity with which calcifications of the aorta, coronary vessels, and the cardiac musculature could be produced. Twenty-four hours after the administration of the irradiated ergosterol, no marked degenerative lesions could be demonstrated, but in the 24 hours following this, the calcification is massive. In the latter author's estimation, the calcification depends on the inability of the serum to retain all of its calcium rather than any preliminary degenerative changes in the recipient tissues. He states that one must visualize a mechanism by which the blood is able to build up a high calcium content and yet be unable to retain it in solution. The formation of a colloidal form of calcium phosphate in blood with a high content of calcium and phosphate may be the key to this. Greenberg and Tufts noted the presence of this colloidal substance in dogs dosed with irradiated ergosterol. However, a difficulty is that Harris and Moore also report tissue calcification in animals kept for long periods of time on large doses of irradiated ergosterol in which the increase of calcium and phosphorus in the blood was only slight.

It has been questioned whether the increase in blood calcium and the toxic effects were actually due to vitamin D itself. Instead, it has been proposed that it was the effect of other materials produced by the irradiation of ergosterol and that the actual vitamin D had no toxic effects in any dosage. A contrary view has been taken by Harris and Moore. Dale, Marble and Marks demonstrated that pure calciferol isolated from irradiated ergosterol has, in excessive doses, the characteristic toxic action on dogs which is found on administration of crude irradiated ergosterol. The same symptoms and physical findings and the marked increase in blood calcium are found in the case of calciferol.

d. Interrelationship between vitamin D and parathyroid hormone. The question as to how vitamin D exerts its action in raising the blood calcium and inorganic phosphate has been actively discussed. The former opinion that these constituents necessarily came from the food is now definitely ruled out. Another proposal which has carried great weight is that the action is an indirect one resulting from either a stimulation of the parathyroid gland to overactivity or by rendering the organism more responsive to the hormone. Much experimental evidence is available which points to an interrelationship between the action of vitamin D and the parathyroid hormone.

Higgins and Sheard found that chicks maintained in the absence of

ultra violet light showed enlargement (hyperplasia) of the parathyroid glands. The addition of 2 per cent of cod liver oil to the diet led to the development and maintenance of more nearly normal glands even when the chicks were not exposed to ultra violet light. Along the same lines, diets poor in calcium induce parathyroid hyperplasia. In rickets and osteomalacia there is hypertrophy of the parathyroid gland.

Important evidence is contained in the work of Morgan and Garrison. Young dogs which were maintained on a diet free from vitamin D and in which the Ca:P ratio was kept within the limits of 1.18 to 1.64 showed but little response in blood calcium to parathyroid administration. Lack of response was independent of the presence of active rickets or other bone disease. Control dogs which had received vitamin D did respond. This also occurred when the animals showed active clinical rickets produced by low phosphorus in spite of the inclusion of vitamin D. They report further that both young and adult dogs fed a diet which contained either a normal Ca:P ratio, a low calcium or a low phosphorus content, and either with or without vitamin D, responded to the administration of parathyroid extract if 0.5 gram per kilo of sodium carbonate was given to make the urine alkaline. The response to parathyroid extract when ammonium chloride was administered was slight. This may not necessarily indicate a failure in the response to parathyroid hormone, but rather a more rapid removal of excess calcium as a result of the ingestion of ammonium chloride. The effect of sodium carbonate was perhaps to delay excretion and hence served to maintain the blood calcium at a higher level.

Hess and his co-workers in certain experiments found that the administration of irradiated ergosterol did not lead to an increase in the serum calcium level of parathyroidectomized monkeys. Similarly, Greenwald and Gross state that 300 to 400 mgm. of irradiated ergosterol did not definitely relieve tetany or raise the amount of blood calcium unless it was supplemented by adding calcium to the diet.

The evidence, however, is by no means entirely in favor of the action of vitamin D through the parathyroids. Hess, Benjamin and Gross found that, while larger doses of irradiated ergosterol were required to raise the blood calcium level in parathyroidectomized rats than in normal animals, in sufficient doses it does so in the absence of the parathyroid gland. Dale, Marble and Marks found that complete parathyroidectomy did not prevent or significantly hinder the fatal intoxication produced by large doses of calciferol and the effect on the level of the blood calcium attained was not marked. Moreover, the histological

pictures of the influence of excess of vitamin D and of the parathyroid hormone on bone are, according to Selye, quite dissimilar. The question offers many difficulties. Since both substances are concerned with bone metabolism, the same yardstick is applied to measure the effects produced by the two. This may readily lead to much confusion and so increase the difficulty of differentiating between specific action of the vitamin or the hormone. More work is required to settle finally the problem.

In the present relation the other vitamins appear to be inactive. It is stated by Bomskov and Seemann that large amounts of vitamin A produce fragility and mineral depletion in bone.

7. THE RELATION OF THE ENDOCRINE GLANDS TO THE METABOLISM OF CALCIUM, MAGNESIUM AND PHOSPHORUS. *a. The parathyroid hormone.* Spontaneous hypoparathyroidism has been reported. The common form of occurrence in human subjects is the result of attempted ablation of the thyroid. Many experimental studies have been carried out on animals following parathyroidectomy. The characteristic reduction in serum calcium following this operation was first reported by MacCallum and Voegtlin and the resulting tetany was attributed by them to the calcium reduction. This explanation has survived many attacks in the passage of time.

Some figures on the blood composition in tetany as the result of parathyroid deficiency are given in table 9. As the results show, there is a marked reduction in the amount of serum calcium and a small increase in the inorganic phosphate. The drop in the amount of calcium occurs largely in the diffusible fraction. The cerebrospinal fluid calcium, on the other hand, is reduced very little below normal. It is the reduction in the amount of diffusible calcium, as will be pointed out, which is probably the significant factor in the onset of tetany. In other conditions where there is also found a reduction in the level of serum calcium as a result of protein reduction, the amount of diffusible calcium remains normal and no symptoms analogous to tetany have been observed. Following parathyroidectomy, it has been shown by Salvesen and Linder that the serum proteins maintain their usual values. In cases of hypoparathyroidism of long standing, considerable decalcification of the bones has been reported. Analysis shows that the bones are low in calcium and phosphorus and relatively high in magnesium. Another paradoxical relation is the development of cataracts of the lenses of the eyes. Lenses with cataract are high in calcium. A striking change in human subjects is found in the teeth which become opaque, brittle,

distorted and generally disintegrated. Partial or complete removal of the parathyroid gland is followed by a defective deposit of dentine in growing teeth, and a defective development of the callus about a bony fracture.

b. Tetany. Mention of tetany has already been made from time to time in this review. At this point it is desirable to discuss the subject in greater detail.

Hyperexcitability of the neuromuscular system which, in the extreme case, is manifested in carpopedal spasm, will serve as a working definition. Tetany can be produced in a variety of ways which may be classified as 1, the result of parathyropriva; 2, from dietary disfunction associated with such diseases as rickets, osteoporosis and osteomalacia; 3, following an alkaline shift in the acid-base balance produced by hyperventilation, overdosage with bicarbonates, continuous gastric vomiting or the injection of alkaline phosphates; 4, according to the work of Kruse, Orent and McCollum, as a result of an extreme deficiency in magnesium. Even now the various forms of tetany readily fall into two groups, one characterized by a reduction, the other with no change in the blood calcium level.

Although, in the words of Grant and Goldman, "Tetany occurs under so many circumstances that it probably cannot be attributed to a single etiological factor," nevertheless, this has not deterred many investigators from attempting to establish a common disturbing factor as the underlying cause of all forms of tetany. The two favorite hypotheses with respect to the etiologic factors responsible for tetany are: 1, all forms of tetany are due to an alkaline shift in the acid-base balance; 2, a lowering in the calcium ion activity of the body fluids is directly responsible for the onset of tetany.

That tetany may result from an alkalization of the body is unquestionable. However, experimental investigation has failed to demonstrate an alkalosis in parathyroid tetany, infantile tetany, and the tetany of healing rickets. At the present time, the alkalosis theory of tetany in its application to the types just mentioned may be considered to have been eliminated.

The difficulty with the theory of a lowered calcium ion activity is to harmonize the types of tetany in which no reduction in the amount of serum calcium takes place. In parathyroid tetany, infantile tetany, osteomalacia with tetany, and the tetany of healing rickets, there unquestionably occurs a lowering in blood calcium content to which the onset of the attacks can be attributed. However, in tetany induced by

an alkaline shift in the acid base balance—except that produced by alkaline phosphate injections—no lowering of the total serum calcium level occurs. Proponents of this theory have belied upon deductions drawn from the Rona-Takahashi equation or modifications of this equation relating the solubility of calcium ion to the pH, the bicarbonate content, and, in later developments, to the phosphate ion content of biological fluids. According to these calculations, an increase in bicarbonate, or phosphate ion, or a reduction of hydrogen ion concentration should decrease the calcium ion concentration and thus lead to tetany. The shortcomings of this equation as a means of estimating the state of the calcium in biological fluids has already been discussed.

Another suggestion that may be made as to the method whereby the calcium ion concentration of the blood might be reduced without a concomitant decrease in the total amount of calcium, is that as a result of the increased alkalinity the plasma proteins are able to combine with more of the calcium to form unionized compounds. This would reduce the concentration of ionized calcium while tending to keep the amount of total calcium high. This hypothesis has been tested by Gunther and Greenberg by determining the amount of diffusible calcium in subjects with tetany induced by voluntary overventilation and others suffering from hysteria. However, no reduction in the amount of diffusible calcium was observed. The reasonable conclusion to accept is that tetany can result from extreme alterations of a considerable number of bodily constituents.

Such reasoning is in harmony with the basic physiological work of the influence of ions in irritability by Ringer, Loeb, etc. From this work there may be set up the relationship,

$$\frac{Na^+ + K^+ + OH^-}{Ca^{++} + Mg^{++} + H^+}$$

as determining the state of irritability. The increase of ions in the numerator leads to an increase in irritability, while the ions in the denominator reduce irritability. Therefore a reduction of the concentration of either calcium, magnesium, or hydrogen ion, as well as an increase of the ions in the numerator, will act to increase irritability. Tetany from this scheme may be the resultant of the alteration of any one of at least six factors.

The ratio of calcium to inorganic phosphorus has been suggested as important in connection with the development of tetany. It seems doubtful as to whether the phosphate is in any way directly concerned.

The actual concentrations of the ions influencing the state of irritability probably outweigh in importance any ratios. More importance has been attributed to the functional value of ratios in the biochemical field than appears to be altogether warranted. In parathyroid tetany with a lowered calcium and somewhat increased phosphate concentration, the calcium to phosphorus ratio is reduced to values of about 1. But one need only consider the blood picture in nephritis to see that this has no particular significance. In this disease when there is a phosphate retention, calcium to phosphorus ratios of 1 or even less are often found, with no indication, however, of a state of tetany.

The exact mechanism by which tetany is brought about has not been elucidated. It is even uncertain which of the tissues, the nerves or muscles, is primarily affected. Martin has argued that tetany is of myogenic origin; that it is due to a disturbance of the internal physiology of muscle. His chief argument is based on the close relation of tetany to myotonia atrophica.

An examination of the composition of tissues has shown no significant change in the content of calcium, magnesium, or phosphate in muscle, nerve, or other tissues of animals suffering from tetany. Experimental changes in the blood composition of these elements, it has also been observed, are not reflected by changes in the tissues, indicating a very considerable impermeability. From this it seems clear that tetany is not the reflection of a deficiency in the amount of either calcium, magnesium, or phosphate in nerve or muscle. Further, the rather wide variation of these constituents found in healthy tissues points to a relative insensitivity of the bodily organs to fluctuations in composition.

Quite the contrary holds true for the blood and tissue fluids. Ordinarily, they are quite constant in composition and only small reductions in the amounts of either calcium or magnesium lead to neuromuscular irritability. Tetany then would appear to be primarily associated with an ion unbalance in the fluids bathing the tissues rather than of a disturbance in muscle or nerve itself. It would appear logical that the unbalance in the fluids alters the permeability and the electric potential of the neuromuscular junctions and thus leads to the heightened irritability and the greater sensitivity to stimuli in tetany. The opposite picture is observed in hypercalcemia which leads to muscular atonia.

Since the state of irritability is a direct reflection of the ionic unbalance of the interstitial fluids, the composition of the blood plasma is a valid measure of the actual state of affairs, rather than, as has at times been suggested, a distorted image of more fundamental changes in nerve and muscle themselves.

Certain qualifications have to be made to this statement. The total amount of calcium or magnesium in the plasma is not a measure of the amount of these elements physiologically available for the maintenance of normal neuromuscular irritability. Since the amount of magnesium which is combined with the plasma proteins is quite small, no great error is involved in the use of the plasma magnesium content as a measure of its physiological activity. However, the analysis of the total amount of calcium in the serum is not always a reliable guide to the state of muscular hyperirritability.

The work of Loeb, Ringer, and others points to the ionic calcium concentration of the blood as the best measure of the physiologically important calcium. Measurement of the calcium ion activity of biological fluids, from what has already been given, is still largely an unsolved problem. The one practical procedure which would at the present time appear to approximate an estimation of the physiologically available calcium is an estimation of the amount of diffusible calcium. From the work on this subject it appears that a reduction of about 2 mgm. per cent in the level of diffusible calcium leads to tetanic symptoms. Liu and Gunther and Greenberg found in human subjects the value of 3.5 mgm. per cent or less of diffusible calcium to be associated with clinical symptoms of active tetany in parathyroproprivic cases, in osteomalacia, and in juvenile cases.

Reed, working on parathyroidectomized dogs, however, came to the conclusion that no relationship can be established between the incidence and severity of tetany and either the absolute amount or the percentage of diffusible calcium. In his estimation, the characteristic feature associated with this type of tetany is the tendency to instability of both the diffusible and non-diffusible calcium fractions.

From recent observations it must be conceded that no one exact value of diffusible calcium can be set as an unvarying tetany level. The organism apparently can in time adapt itself to some extent to different levels of calcium and reductions from the stabilized level results in tetanic symptoms. This has been strikingly brought out by the sequence observed following operations on cases of hyperparathyroidism. It would appear that a lowering of 2 to 3 mgm. per cent in the concentration of diffusible calcium from the accustomed level leads to an immediate onset of tetany.

It also appears possible that other as yet unknown factors may outweigh the hyperirritability effect of a low diffusible calcium. A well characterized example of this is the parturient paresis of cattle studied

by Sjollema and Seekles. The average amount of diffusible calcium is stated by them to be about 2.0 mgm. per cent. Animals with this disease, contrary to what would be expected, are quite insensitive to such stimuli as needle pricks.

The relation of magnesium to tetany is set forth by the work of Kruse, Orent and McCollum. These authors have demonstrated that a most dramatic syndrome of symptoms terminated by early death follows the rearing of young animals on a practically magnesium free diet (1.8 parts per million). The striking blood chemical changes found in the dog on such a diet were an early reduction in serum magnesium content to about 0.5 mgm. per cent. This drop in serum magnesium was shortly followed by a marked increase in the amount of total cholesterol with a commensurate decrease in fatty acids, so that the total fat content remained constant. The increased amount of cholesterol is found to consist mainly of cholesterol esters.

A natural form of magnesium tetany is the grass tetany of cattle also studied by Sjollema and Seekles. In this disease both the amount of calcium and magnesium are reduced, the average calcium value being about 6.7 mgm. per cent and the magnesium about 0.5 mgm. per cent, with values seldom over 1 mgm. per cent. However, the level of diffusible calcium on the average is fairly high, viz., 4.2 mgm. per cent. These animals are nervous and restless, show anorexia and muscle twitching and finally develop tonic-clonic convulsions with involvement of the limbs. The seizures are followed by a comatose state. Sjollema favors intoxication as the cause. The basic cause of this disease is unknown since the diet would appear to contain ample amounts of magnesium.

c. Hyperparathyroidism. The isolation of active extracts of the parathyroid hormone by Collip and by Hansen made possible the experimental study of the properties of this secretion. The knowledge gained from such studies laid the foundation for the discovery of clinical hyperparathyroidism exemplified in the disease, osteitis fibrosa cystica.

The most striking effect of the injection of parathyroid extracts into animals is the increase in the amount of blood calcium. The response is not uniform in all animals. Dogs very readily show a calcium increase and so do human subjects. On the other hand, the cat, the rabbit, the guinea pig, and many other animals show only a slight increase in the amount of blood calcium. Subcutaneous or intramuscular injections are the most satisfactory. Intravenous injection is said to reduce the time required for the onset of the calcium increase to about 4 to 6 hours,

but the increase produced in the calcium level is lowered by about 50 per cent. With the previously mentioned routes of administration, Collip and co-workers state that it requires about 12 to 18 hours to reach the maximum increase in the blood calcium content of dogs. Greenberg and Gunther observed that about 24 hours were required to produce this effect in their animals. With single doses the rise in calcium is in general proportional to the dosage employed.

Among the first observable effects of the injection of parathyroid extract is a small rise in serum potassium and magnesium level. The magnesium rise has its onset in 2 to 4 hours following the injection and disappears long before the maximum of the calcium increase. The amount of magnesium increase, as shown by the work of Greenberg and Mackey, averaged about 0.6 mgm. per cent.

Bulger and Gausmann found in clinical cases of hyperparathyroidism, on the other hand, a somewhat lowered amount of serum magnesium. These authors found that there is a negative magnesium balance in the hyperparathyroid state, and that individuals recovering from hyperparathyroidism store magnesium. In their estimation, the influence of the parathyroid hormone on magnesium metabolism is probably ancillary to its action on calcium or phosphorus metabolism.

Discussion as to the mechanism of the action of the parathyroid hormone has elicited a number of theories. The first views, based on the assumption that the hormone causes the elaboration of a citrate-like compound which maintains the level of the calcium in the plasma by increasing the solubility, it would appear, will now have to be abandoned.

In the opinion of Albright, Bauer, Ropes and Aub, the primary effect of the parathyroid hormone is on phosphorus rather than on the calcium excretion. They state that the first metabolic changes, when the extract is administered, are an increase in phosphorus excretion and a fall in serum phosphorus. When the treatment is discontinued, the converse is true. This effect, it is assumed, is brought about by lowering the renal threshold for inorganic phosphate. This leads to a decrease in the amount of inorganic phosphate in the blood which, in turn, results in an increase of the serum calcium content.

Selye has shown that the parathyroid hormone actively stimulates osteoclastic changes in the bone. Furthermore, it is generally conceded that the increase in the amount of serum calcium and urine calcium takes place principally at the expense of the calcium stores in the bones. In the viewpoint of Thomson and Collip and Thomson and Pugsley, the primary action is upon the bone tissues themselves and any changes

in blood or urine are only a resultant. This explains the difference in effect found among different animals. The dissolved bone salts thrown into the blood stream, if they can be sufficiently rapidly excreted, will change the blood composition but little. Certain animals apparently are able to do this. In others, such as the dog, there is a retardation of the calcium excretion with a consequent rise in the amount present in the blood.

According to Thomson and Pugsley the rise in the concentration of serum calcium following upon the intravenous injection of parathyroid extract is not necessarily accompanied or preceded by a decrease in the amount of inorganic phosphate. Moreover, they point out that a decrease in phosphate content produced by the administration of glucose and insulin leads to only a minor increase in serum calcium. Greenberg and Gunther observed a marked drop following subcutaneous injection of parathormone in dogs with an initially high phosphate content, but, on the other hand, no decrease in the amount of phosphate was observed in animals with an initial value of 4 mgm. per cent or less.

In the estimation of the writers the last two mentioned theories are not mutually exclusive. The histological evidence would appear to point to action on bone itself as the primary effect of the hormone. However, an action on the kidney causing an increased excretion of phosphate is also strongly indicated. Otherwise, if the idea of a demineralization of the bone is accepted, inorganic phosphate, as well as calcium, should accumulate in the blood stream with an overdosage of the hormone. The difference in action between excess vitamin D and parathyroid extracts also favors the idea of an action on the kidney. Administration of vitamin D, which also causes withdrawal of calcium from the bones, produces no reduction in the amount of plasma phosphate as the calcium level increases, but, on the contrary, there usually is some accompanying increase in the concentration of phosphate.

There is some evidence that other tissues, perhaps the liver, may intermediate the action of the parathyroid hormone. Both Greenberg and Nitzescu have observed that liver poisoning by phosphorus prevents the calcium increasing response of parathyroid extracts in dogs. Thomson and Collip suggest that this effect may be due to injury to bone cells by the phosphorus. In as yet unpublished experiments, Greenberg has found that certain other liver poisons—namely, carbon tetrachloride and hydrazine—do not show the effect found with phosphorus. On the other hand, the attested bone marrow poison, benzene, was also without effect in preventing a rise in the concentration of serum calcium.

Repeated injections of considerable amounts of parathyroid extract within intervals of about 6 hours lead to an early fatal result. The level of serum calcium is elevated to perhaps as high as 20 mgm. per cent. More important is the fact that the content of inorganic phosphate of the plasma also becomes markedly increased. The at first marked excretion of calcium, phosphorus, and nitrogen in the urine absurptly declines. At this time, about 36 hours after the initial dose, the level of serum calcium falls off somewhat; the amount of non-protein nitrogen and urea increase, as well as does the phosphate. This latter fact is to be attributed to failure of renal excretion as a result of kidney damage. Physically, the animals show anorexia, diarrhea, vomiting and lassitude, which are followed by muscular atonia, coma, and death. The blood becomes very viscous and the volume markedly decreases. There is every sign of dehydration, failing circulation, and terminally a marked acidosis. The calcium content of the soft tissues, particularly the kidneys, is increased. Histological evidence of calcification is readily demonstrated. Collip showed that this pathological picture can be reproduced by successive intravenous injections of calcium salts and of acid sodium phosphate.

Greenberg and Tufts found that when an increase of only about 2 mgm. per cent is produced in the content of serum calcium by parathyroid extract, the rise occurs almost wholly in the diffusible calcium fraction. When the serum calcium increase is greater than this, the rise is about equally divided between the diffusible and the non-diffusible calcium fractions. The work of Greenberg and Tufts indicates that most of the increase in the non-diffusible calcium is due to the formation of a colloidal calcium phosphate. Andreyev and Pugslely observed a marked effect on the calcium content of the saliva from the parotid gland on administration of parathormone or of irradiated ergosterol.

Other endocrines. While the parathyroid gland has been most prominently connected with the metabolism of the alkali earth elements, many attempts have been made to invoke the influence of other endocrine secretions. The most conspicuously successful have been Aub and his associates, who demonstrated a greatly increased calcium and phosphorus catabolism in hyperthyroidism. Of the other glands, the thymus particularly has come in for a good deal of attention. The prominence of this gland in childhood and its involution after adolescence early suggested the possibility of a relationship between it and skeletal growth. Changes of this gland in conditions with upsets in calcium metabolism have been reported. Chemical attempts have also been

made to isolate active extracts. Nitschke reports the preparation of two active and specific thymic substances, one of which had a blood calcium and the other an inorganic phosphate lowering effect. Both are said to have produced tetanic convulsions. The calcium lowering principle is described as water soluble and heat, acid, and alkali resistant. The potency is lost by dialysis. The phosphate lowering principle is described as being a stable fatty-like substance. The presence of these hormone-like substances, according to this and other authors, is not limited to the thymus gland, but is generally present in all lymphoid tissue. While a good deal of work has appeared on the subject, it has not led to a definite assessment of the value or the reality of the thymic substances. Similar acting substances are claimed to have been isolated from the spleen.

A relation between the sex glands, anterior pituitary, and bone salt metabolism has also been sought. The follicular hormone is said to increase the serum calcium concentration of castrated animals. Cannavo and Indovina state that prolan markedly increases the content of serum magnesium, but does not alter the balance or composition of the tissues. In a recent review Evans states: "There seems no question but that we have to do with disturbance of calcium metabolism after hypophysectomy, as reflected in the decreased calcium content of the blood. . . . ." In the disease, acromegaly, with an overactivity of the hypophysis, there are marked alterations in the skeleton. Similar changes have been produced experimentally with the growth promoting principle of the pituitary. A comprehensive review of this topic by Zwarenstein has recently appeared.

8. Excretion of calcium, magnesium and phosphorus. A clear understanding of the metabolism of the three elements under consideration requires that not only the paths of absorption and excretion be known, but also that the factors which influence both processes be clearly defined. This is not easily done, since excretion of these substances takes place both in the urine and the intestines. Moreover, the factors which may play a rôle in determining the amounts which are quantitatively excreted in each channel probably vary with different animal species. Thus in the herbivora, calcium phosphate plays a very important rôle in maintaining the necessary constancy in the acid-base equilibrium, while in carnivora and omnivera, this factor is probably a secondary one. It is not surprising then that the inorganic phosphate of sheep urine, according to Watson, may fall to 1 to 2 mgm. per cent when the animals are maintained on a pasture or chaff diet. The

amount excreted was independent of the concentration of inorganic phosphorus in the blood over a wide range of concentrations. It was further noted that, although there is a renal threshold for inorganic phosphate above which it is secreted into the urine, the threshold level in sheep is variable both in different sheep and in the same sheep under different conditions. According to Brain, Kay and Marshall and Walker and others, in rabbits and in man, a relation exists between the rate of excretion of inorganic phosphorus in the urine and its concentration in the blood. The correlation coefficient for young adult humans is 0.416 $\pm$0.071. The source of inorganic phosphorus in the urine is the inorganic plasma phosphorus and apparently not the organic phosphorus, at least not until this form has been split to inorganic phosphorus. The renal threshold for man is about 2 to 3 mgm. of phosphorus per 100 ml. of blood. Below this level excretion practically ceases or is a minimum.[7] The inorganic phosphorus in the urine of young adults after fasting is normally 0.026 to 2.10 with a mean of 0.603 mgm. per ml. The organic phosphorus varies between about 0 and 0.195 mgm. per ml. In leukemia, the amount of organic phosphorus which is excreted in the urine may increase from the normal range of 6 to 13 mgm. to values as high as 23 mgm. per day.

Unfortunately, the amount of each of the three elements which appears in each of the excretory channels does not constitute an index of either endogenous or exogenous metabolism. Both urine and feces probably contain both endogenous and exogenous fractions. Moreover, a portion of each of the three elements, especially phosphorus, may be excreted in the upper levels and reabsorbed in the lower levels of the intestinal tract. Two types of tissues contribute to the endogenous outputs, i.e., soft and skeletal. Phosphorus, for example, is present in both types of tissues in large amounts, while the major portion of calcium is present in the skeleton. The endogenous output, even if measurable, would not serve to distinguish the metabolism of one type of tissue from another since it is a composite of both. In view of the foregoing, it is not surprising to note the fragmentary status of the subject.

[7] According to unpublished data of Goss, cattle on low phosphorus rations which have a blood phosphorus level of about 3.5 mgm. per cent excrete 1 to 1.5 mgm. per 100 ml. of urine (200 mgm. per day). The control animals with a blood phosphorus level of 8 to 9 mgm. excrete 10 to 15 times as much phosphorus in the urine as the first named group of animals. The calcium figures are just reversed, the low phosphorus animals excreting larger amounts of calcium in the urine than the control animals.

An ingenious method has been employed by Bergeim to study both the absorption and excretion of calcium and phosphorus in the intestines of rats. He fed them a quantity of iron oxide which is practically unabsorbed and is quantitatively eliminated in the feces. At different levels of the intestinal tract he withdrew samples of the contents and determined the ratio of calcium and phosphorus to iron. Both rachitic animals absorbed calcium from the small intestines. The animals which received cod liver oil showed a positive balance throughout the small intestines, while in rachitic animals, the absorbed calcium was largely excreted in the lower intestinal levels and the balance was negative. Both groups of animals showed considerable excretion of phosphate into the upper intestinal tract. This was reabsorbed in the lower intestines by the normal animals, but not by the rachitic rats.

While the larger part of the calcium which is excreted appears in the feces, wide fluctuations in both excretory channels have been recorded. Nelson and his associates carried out their experiments on men whose ages ranged from 13 to 70 years, and who were maintained on mixed diets. The total daily calcium (as CaO) excretion varied from 0.588 to 1.420 grams. The urinary fraction contributed 0.175 to 0.619, while the fecal excretion represented 0.413 to 0.801 gram. The urinary magnesium (as MgO) ranged from 0.191 to 0.313 gram. Of 25 individuals, 17 excreted more calcium than magnesium and 8 excreted more magnesium than calcium in the urine. Fluctuations in the ratio of urinary to fecal calcium, varying from 1:2 to 1:1, have been recorded. Such variations undoubtedly are in large part influenced by the amount of insoluble calcium compounds which are contained in the diet. The larger portion of the phosphorus excreted by man appears in the urine. Of a total daily output of 1.41 grams of phosphorus recorded by A. E. Taylor, 1.11 grams represented urinary phosphorus and the balance fecal excretion. A strict correlation between nitrogen and phosphorus excretion is not possible since the amount of phosphorus which is excreted will in part depend on the amount of nucleoprotein, as well as of phospholipids, contained in the diet. When an individual is fed meat, the time sequence in the excretion of sulfur, nitrogen, and phosphorus takes place in the order in which the elements are mentioned.

Some attempts have been made to estimate the endogenous outputs of calcium and phosphorus in humans. On diets which contained no calcium, the fecal excretion of this element falls practically to zero, while urinary excretions, ranging from 0.43 to 0.87 gram (as Ca), have been recorded. On the thirty-first day of the fast by subject L, studied

by Benedict, and quoted by Lusk in *The Science of Nutrition*, the urinary excretion of calcium was 0.138 gram, while during the initial period of three weeks, the output ranged from 0.217 to 0.237 gram. The daily output of urinary P_2O_5 during the initial three weeks was approximately 1.65 grams. This fell to 1.32 grams on the thirty-first day of the fast. The urinary output of magnesium on the first day of the fast was 46 mgm. This rose to 72 mgm. on the eleventh day and then fell to a constant value of 53 mgm. from the twenty-first to the thirty-first day. It is difficult to state whether endogenous fecal excretion of calcium, magnesium, and phosphorus occurs since the possibility of reabsorption, if intestinal excretion occurs at lower levels, must be ruled out. Aub and his associates have shown that the amount of calcium which was excreted by a pregnant woman on a low calcium diet was essentially normal during gestation and was not available for the requirements of the fetus. This is indicative that endogenous calcium is not available for fetal requirements. The endogenous output of calcium will undoubtedly vary somewhat with the amount which is stored in the skeleton. The previously mentioned experiments of Goss on rats show that the output of calcium is extremely low when animals are placed on low calcium diets and their skeletons have been severely depleted by repeated pregnancies. The endogenous output of phosphorus represents not only the metabolism of the skeleton whose reserves may be markedly depleted without serious injury to the animal, but also that of nucleic acid phosphoproteins and phospholipins which are essential constituents of the vital organs and of the nervous system. The latter stores probably cannot be considerably depleted without serious consequences to the life of the animal.

Various factors may influence the excretion of the three elements. The ingestion of potassium salts, including potassium citrate, has little or no influence on calcium excretion. Bogert and Kirkpatrick report a decided diminution in urinary calcium when individuals were placed on base-forming diets, while the reverse condition was noted in the case of acid-forming diets. According to Aub and his co-workers, the excretion of calcium is but comparatively little affected by acid and alkali. On a low calcium diet, the fecal calcium remained nearly constant despite great changes in potential acidity. In a case of steatorrheic tetany with low serum calcium, the administration of a high calcium diet plus calcium and ammonium chloride failed to cause an increase in urinary calcium. The urinary excretion is within limits apparently independent of the reaction. Ammonia plays a much more important

rôle in neutralizing fixed acid when the latter is ingested than calcium, although some increase in urinary calcium may take place when the dose of fixed acid is sufficiently high. Ingestion of fixed acid or alkali undoubtedly influences somewhat the amount of calcium and phosphorus which appears in the urine. Thus Zucker records a decrease in urinary phosphorus from 5.36 to 4.52 grams on ingestion of a daily dose of 15 grams of sodium bicarbonate and an increase to 5.60 grams when 300 cc. of N/10 NCl were ingested. The corresponding amounts of calcium which were excreted in the urine were 1.27, 0.99, and 1.41 grams. The recorded variations are not commensurate with the amounts of acid and alkali ingested and indicate that calcium and phosphorus play a minor rôle in neutralizing fixed acid or alkali in man. Large variations in the intake of phosphorus were found to be without material influence on the output of urinary or fecal calcium. (Low phosphorus diets may have some influence on the excretion of fecal calcium). On the other hand, large doses of calcium, magnesium and other salts which form insoluble phosphates will materially reduce the amount of phosphorus which is excreted in the urine. Schmidt and Hoagland have recorded an experiment on a pig in which the urinary phosphorus excretion was reduced from 150 to 10 mgm. by administration of aluminum hydroxide. Fecal calcium is increased when the amount of crude fiber in the diet is increased and falls when the individual is placed on a high protein diet. This may be accounted for by the fact that the amount of calcium which is set free and becomes available for absorption depends in large part on the ability of the body to digest the food and thus set free the combined calcium. A characteristic of nephritics is the low urinary calcium excretion. A figure of 30 mgm. in a 5 day period has been reported.

Approximately one-fifth to one-half of the output of magnesium salts in infants and adults is excreted in the urine, while the major portion is eliminated along with calcium and phosphorus in the feces. The administration of calcium chloride leads to a negative magnesium balance, presumably due to the acidity of the ingested salt. While a marked increase in the retention of calcium and phosphorus on addition of cod liver oil to the diet of infants maintained on breast and on cow's milk was noted by Swanson, the effect on the retention of magnesium was less. In fact, there was a decreased retention of magnesium when cod liver oil was administered to an infant fed cow's milk.

According to Pugsley the injection of parathyroid hormone leads to an increase in the fecal excretion of calcium and of urinary phosphorus.

An increase in the excretion of calcium in the urine may also occur, but this is not long maintained. An increased fecal excretion of calcium was also noted by Greenwald and Gross in dogs which had received parathyroid hormone for short periods of time. On the other hand, Albright and others did not observe any such effect.

Hunter demonstrated that administration of parathyroid hormone to patients already in negative calcium balance markedly increased the loss of calcium and this loss was accounted for by an increased urinary excretion. In one patient, as a result of large doses of parathyroid hormone, the urinary calcium rose from 0.069 to 0.754 gram in a 3-day period. Parathyroid hormone was found to increase abruptly the phosphorus excretion in the urine without affecting fecal excretion. Discontinuance of the treatment caused the urinary excretion to fall to a level below normal. The increased excretion of phosphorus is greater than can be accounted for by assuming that it is due to liberated calcium phosphate. The excess is apparently derived from the soft tissues. The above facts have been interpreted to mean that the primary effect of parathyroid hormone is on phosphorus metabolism. On continued administration of the hormone an opposite effect is produced.

Pugsley and Selye have shown a marked increase in the calcium excretion of rats which had received daily injections of parathyroid hormone for a period of 4 days, but the excretion returned to normal or subnormal within 8 to 10 days despite continued injections. A so-called "immunity" to parathyroid hormone was established. These facts elucidate to a considerable degree the action of this substance. The first response histologically is the formation of numerous osteoclasts with rarefaction of the bones which is expressed in terms of an hypercalcemia and increased calcium excretion. The second stage is the disappearance of the osteoclasts which coincides with the return of the previously increased calcium excretion to normal. This is followed by the appearance of numerous osteoblasts with bone apposition proceeding to such an extent that the picture of "marble bone disease" can be experimentally produced. If this takes place sufficiently fast, there may be a decreased calcium excretion. According to Hunter and Aub, administration of parathyroid hormone to patients with chronic lead poisoning is followed by an increased excretion of both calcium and lead. Taylor and Fine have shown that the excretion of injected calcium is several times greater in parathyroidectomized than in normal animals. In normal cats, the amount of fecal calcium is small but constant. The administration of parathyroid hormone was without sig-

nificant effect over a 6-hour period. Studies over extended periods were not carried out. Bulger and Gausmann have shown that in hyperparathyroidism there is a negative magnesium balance. The effect of the hormone on magnesium metabolism is secondary to that on calcium metabolism.

Daily oral administration of large amounts of irradiated ergosterol (20,000–50,000 units) leads to an increase in urinary calcium and to a decrease in fecal calcium and phosphorus. This effect can also be obtained in rats which are immune to parathyroid hormone. Magnesium has apparently received no consideration in this connection. Warkany finds that administration of irradiated ergosterol to rabbits is followed by a large increase in urinary phosphorus. Taylor and Weld have shown that large doses of irradiated ergosterol and of parathyroid hormone have quite similar effects when administered to dogs. The post-mortem findings in the gastro-intestinal tract are identical. Both lead to a rise in serum calcium, decalcification of the bones, and an increased urinary calcium excretion. The effect of irradiated ergosterol on calcium and phosphorus excretion obviously will depend on whether the amount administered is optimal, a moderate overdosage, or an extreme over-dosage. Doses of irradiated ergosterol, as large as 4,000,000 units, have been administered daily to rabbits.

Aub and his associates have established the fact that administration of thyroid substance leads to an increase in the excretion of calcium. Patients with exophthalmic goiter and those with hyperfunctioning adenomata showed a marked increase in the output of calcium and phosphorus in both urine and feces. This is apparently not directly associated with an increased tissue metabolism, since in fever or leukemia increased calcium excretion was not noted. It is more probable that both calcium and phosphorus are derived from the bones.

In steatorrhea, a considerable amount of calcium appears in the feces as calcium soap. This has been attributed to failure of vitamin D absorption and hence to faulty calcium utilization. The failure to absorb vitamin D may in part be due to excess amount of unabsorbed fat or possibly a paucity in bile flow or bile acid secretion. While there can be no denying that failure to absorb vitamin D would prevent calcium utilization, a large amount of unabsorbed fatty acid leading to the formation of calcium soaps would probably also hinder absorption of calcium.

9. Calcium, phosphorus and magnesium balances. It is obvious that the prerequisite to a metabolic balance in the three elements is the

maintenance of the status quo. This is realized only in normal, well nourished individuals. In growth there is obviously storage of the three elements. Quantitatively this depends on the input and the rate of growth. As has already been stated, Sherman and Hawley's experiments on children ranging from 3 to 13 years of age showed a daily storage of 10 mgm. of calcium and 8 mgm. of phosphorus per kilo of body weight.

Some of the most extensive balance studies on humans have been reported by G. W. Clark. From a quantitative standpoint the balances depended in large part on the previous nutritional history of the individual. One adult stored 53 grams of calcium, 79 grams of phosphorus, and 3.7 grams of magnesium over a period of 28 weeks. The largest amounts stored in one week were 3.9 grams of calcium, 3.4 grams of magnesium, and 6.2 grams of phosphorus. Curiously, during a number of weekly periods, this individual was in negative magnesium balance despite the fact that the balance in the other two elements was positive. Another individual, on the same diet, stored over a 19 week period 3.5 grams of calcium, 25.3 grams of phosphorus, but lost 6.9 grams of magnesium. The large amount of phosphorus stored represented storage of soft tissues since the amount of nitrogen stored during that time was 346 grams.

Reference has already been made to the work of Forbes, Hart and Steenbock, and Goss and Schmidt showing that during periods of heavy lactation, cows, rats, and probably other animals are in negative calcium and phosphorus balance despite adequate intakes. The data by many other workers are essentially in accord with these statements. Quantitatively, the loss will in large part depend on the amount of milk secreted. However, when the milk flow is not high and the diet is supplemented with both green grasses and calcium salts, the negative balance may be minimized or even changed to a positive balance. In an experiment carried out on a cow by Hart, Steenbock and co-workers, the average daily excretion of calcium was as follows: urine, 2.8; milk, 23.4; feces, 111 grams. The average daily input was 142 grams. The average daily storage was about 5 grams of calcium. Another balance experiment on a cow was carried out by Miller, Yates, Jones and Brandt. The daily partitions of calcium, magnesium, and phosphorus respectively were as follows: feed, 117, 45, and 58.5; milk, 27.3, 2.8, and 22.3; urine, 0.8, 8.5, and 0.7; feces, 95.1, 36.0, and 36.7 grams. The balances were: calcium, −6.2; magnesium, −2.3; and phosphorus, −1.2 grams.

Hunscher found negative calcium and phosphorus balances in women

during early lactation in spite of large intakes. Usually, but not invariably so, both calcium and phosphorus are stored during pregnancy.

As has already been pointed out, the balances of the three elements are determined, not only by adequacy of intake and the previous condition of the individual with respect to the amounts of the three elements stored, but also by the amount of vitamin D ingested, the amount of parathyroid hormone secreted or administered, the presence of bile in the intestines, the activity of the thyroid gland, and by whether the individual is pregnant or lactating.

In general it may be stated, by way of résumé, that storage of the three elements, provided the diet is adequate, takes place in growth or when individuals have for some time been maintained on a diet deficient in these minerals. Vitamin D increases retention in the young, while in the adult, daily doses of 5 to 30 mgm. of irradiated ergosterol, while not affecting the balances, may lead to a shift of calcium and phosphorus from feces to urine. In experimental hyperparathyroidism there is a decreased retention of calcium and phosphorus, while after parathyroidectomy these balances increase but diminish on parathyroid administration. Administration of parathyroid hormone to normal animals in most instances is followed by a prompt increase in the urinary output of calcium and phosphorus. The fecal excretion of calcium is also increased, but the phosphorus is less affected. Extirpation of the parathyroid gland leads to a considerable reduction in urinary calcium and, to a lesser extent, fecal calcium also. The excretion of phosphorus is likewise diminished. In human patients with hyperparathyroidism an excessive urinary excretion of calcium takes place. The phosphorus output may or may not be parallel to this. In hyperthyroidism both urinary and fecal calcium and phosphorus are above normal, while in myxedema the converse is true. Administration of thyroid substance in the latter condition is followed by an increased excretion. In pregnancy, storage of the three elements takes place, while in lactation the calcium and phosphorus balances are usually negative.

It should be stressed that the information which is obtained from balance experiments represents the summation of processes which take place continuously over the period of time included in the balance, and that a more definite index of these processes at any moment can usually be obtained by a study of the blood picture. A difficulty which may at times be encountered in using the blood picture index is the variability in blood constituents of different animals and also in the same individual at different times under apparently normal conditions. Perhaps this

is a reflection of the many variables which are concerned. The best picture is probably obtained by simultaneous determinations of blood levels and balance data.

In osteomalacia and osteoporosis, the calcium balances are decidedly negative. The same thing may be at times found in coeliac disease and in idiopathic steatorrhea. Shohl and Bennett report that the calcium balances in puppies maintained on a rickets producing diet were positive, but less than normal, while the phosphorus balances were still more deficient and may even be negative. Even when the phosphorus was high and the calcium low, the most marked deficiency lay in the retention of phosphorus. On restricted intakes of rickets-producing diets, the metabolism of rats was not essentially different from that of the animals which had free access to the diet. Steenbock and his co-workers, Kletzien, Templin and Thomas, have shown that administration of vitamin D to rats maintained on a high calcium-low phosphorus or low calcium-low phosphorus ration markedly reduces the losses of the two elements. The calcium content of the newborn was fairly constant irrespective of the additions to the mother's diet.

In the early stages of nephritis, the calcium output in the urine is decreased, while the output of phosphorus remains at a normal level. In later stages, the excretion of this element may also drop. In renal rickets a disturbance in endogenous calcium and phosphorus metabolism apparently occurs as a result of chronic nephritis which apparently leads to phosphorus retention. In four cases of Paget's disease, one of bone cyst, and one of bone sarcoma, Scriver and Venning found no deviation in calcium metabolism from the normal and there was no evidence of parathyroid dysfunction. The factors concerned may in these conditions operate so slowly that their influence may escape detection by analytical methods. A marked retention of calcium has been noted in calcinosis universalis. A recent review of pathological calcification has been made by Barr.

10. MISCELLANEOUS. *a. Egg production and development of the chick embryo.* The laying hen is of particular interest from the standpoint of calcium metabolism The calcium turnover due to eggshell formation is high. The shell of an average hen's egg contains about 5.5 gms. of calcium carbonate. Added to this are the requirements of calcium and phosphorus, for the liquid portion of the egg. Both calcium carbonate and gluconate as well as calcium citrate can be used for egg production, the latter being slightly more efficient. The hen uses both skeletal and ingested calcium for egg formation. The skeletal calcium is withdrawn

mainly from the softer bones. In this respect the hen behaves in a manner analogous to the lactating cow. The formation of soft shell eggs cannot be ascribed to a lack of calcium since feeding of calcium lactate in such cases has not led to increases in shell thickness. The thymus is apparently not related to egg-shell formation.

According to Buckner and his collaborators, laying hens whose supply of calcium is limited will continue to lay eggs until there is a general depletion of magnesium, phosphorus, and calcium in their bones and carcasses. As long as there is eggshell formation, the contents of the egg with respect to the three elements remain fairly constant. A gradual thinning of the egg shells may take place. A calcium deficient diet may materially decrease the number of eggs laid. Such a diet may materially diminish the calcium and phosphorus in the leg bones and lead to depletion of body material and general vigor. Hens which were fed a ration containing corn, buttermilk, and limestone produced larger eggs than those which received corn, mash containing tankage, and limestone. Buttermilk appears to be an excellent ingredient in the ration for egg production as well as for general physical well-being.

Russell and McDonald have shown that during egg production more phosphorus is excreted in the droppings than during non-production and more than is necessary to form tricalcium phosphate with the excretory calcium, suggesting a metabolic change involving phosphorus compounds during egg production. During non-production the percentage of phosphorus retained is greater than that of the calcium, and the phosphorus in the droppings is less than that required for the formation of tricalcium phosphate. The ratios of calcium to phosphorus in the droppings of laying and non-laying pullets were lower during the period of egg production.

Magnesium cannot replace calcium in the shell, but strontium can to a certain extent be so used.

Egg production in the hen is accompanied by a marked increase in calcium and phosphorus of the blood. Magnesium, potassium and sulfur are slightly increased and chlorine is diminished. According to Charles and Hogben, when an egg is present in the oviduct, the calcium content of the serum varies from 10.5 to 28.5 mgm. per cent. Apparently the active ovary maintains the blood calcium at a higher level than that of the sexually inactive or immature bird. The presence of the egg in the oviduct is accompanied by the mobilization of calcium in the tissues during shell secretion which is reflected by a rise in the level of serum calcium. There is also a similar rise in the concentration of magnesium.

As early as 1822, Prout showed that the chick embryo at the time of hatching contained more calcium at the beginning of incubation than the entire egg excluding the shell. The extra calcium was derived from the egg shell. The liberation of carbon dioxide by the chick embryo influences the solubility of calcium carbonate of the egg shell by converting it into bicarbonate, thus rendering the calcium available for the needs of the embryo. Adequate provision for the phosphorus requirements of the chick embryo is contained in the yolk in the form of phospholipins.

b. Dental aspects. In recent years, the teeth have received considerable attention with a view to elucidating the factors which are concerned in the synthesis of these important structures. The problem is a complex one not only from this standpoint, but also from the standpoint of incidence of dental caries and pyorrhea. The subject is so large that it cannot properly be included within the scope of the present article. For comprehensive discussions, the reader is referred to articles by Mellanby, Marshall, Höjer, Hanke, Bunting and others. While all of the factors have by no means been elucidated, there appears to be sufficient evidence to indicate that dietary factors play a very important rôle in dentition through the life cycle of the individual. Among these may be mentioned adequacy in the intake of calcium, phosphorus, and the fat soluble vitamins A and especially D. Cereals and cereal products, as well as foods promoting growth without having a calcifying influence, decidedly hinder normal tooth formation. The tissues in which the teeth are embedded must also be adequately nourished and for this purpose vitamins C and A are especially important. Due to their exposure, teeth, unlike other hard tissues, are subject to wear and to bacterial infection either directly or indirectly as the result of acid production in the mouth. The entire story of the care of the teeth from the nutritional, mechanical, and dental standpoints has still to be written.

From the foregoing it is evident that there are many gaps which must be filled in before our knowledge of the subject of calcium, magnesium, and phosphorus metabolism can be considered as being fairly well established. Too much emphasis has been placed on the metabolism of each individual element rather than consideration being given to the subject in its entirety and thought being directed simultaneously to all of the various factors which enter into a comprehensive understanding of the subject. Not enough emphasis has been placed on the multiple functions which are played by each of the elements. As usually carried out, a metabolic experiment represents the integrated whole and not a differ-

entiation of each of the functions which the individual element performs in the various types of tissues present in the animal organism. More consideration must be given to the fact that the mechanisms in which the three elements enter probably vary in different animals and a direct comparison of data from various organisms may not always be valid. The chemical makeup of parathyroid hormone is still to be determined. Its mode of action is in many respects unknown. The same statements apply to vitamin D. The knowledge of the part played by other endocrine glands with respect to the three elements is still fragmentary. The newer methods of differentiating the various calcium, magnesium, and phosphorus compounds in blood must be more extensively and intensively applied to the study of the rôles which these elements play. Magnesium has especially been neglected in metabolic studies. Further work should be directed to the possibility of determining whether the rate of calcium and phosphorus absorption can be increased so as to place lactating animals in equilibrium. Finally, thought should be given to the question as to whether, in such slow progressing conditions as Paget's disease, as well as in other pathological conditions, more accurate criteria than those which come within normal variability may be used for diagnosis at an early stage of the condition.

The subject which has been reviewed is full of possibilities for the investigator who approaches it with an adequate training, technical skill, patience, persistence, and above all, an open mind.

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GENERAL REFERENCES

AUB, J. C. Calcium and phosphorus metabolism. Harvey Lectures **24**: 151, 1928.

BARR, D. P. Physiol. Rev. **12**: 593, 1932.
Am. J. Cancer **16**: 1424, 1932.

BLUNT, K. AND R. COWAN. Ultra violet light and vitamin D in nutrition. Chicago, 1930.

BUNTING, R. W. Science **78**: 419, 1933.

CANTAROW, A. Calcium metabolism and calcium therapy. Philadelphia, 1931.

COLLIP, J. B. Medicine **5**: 1, 1926.
West. J. Surg., Obst. and Gynec. **40**: 602, 1932.

COWGILL, G. R. Dental Cosmos **76**: 223, 1934.

FORBES, E. B. AND M. H. KEITH. Ohio Agric. Exp. Station, Tech. Series Bulletin no. 5, Wooster, Ohio (1914).

GOLDBLATT, H. Ergebn. allg. Path. Anat. Menschen u. Tiere. **25**: 58, 1931.
GYÖRGI, P. Handb. Normal. Path. Physiol. **16**: 1555, 1931.
HARRIS, L. J. Lancet **1**: 1031, 1932.
HESS, A. F. Rickets including osteomalacia and tetany. Philadelphia, 1929.
Rickets, osteomalacia, and tetany. London, 1930.
HEUBNER, W. Handb. Normal. Path. Physiol. **16**: 1416, 1931.
HOWLAND, J. Medicine **2**: 349, 1923.
HUNTER, D. Quart. J. Med. **24**: 393, 1931.
Lancet **218**: 897, 947, 999, 1930.
IVY, A. C. Physiol. Rev. **14**: 102, 1934.
JAFFE, H. L. Arch. Path. **16**: 63, 112, 236, 1933.
Arch. Path. **15**: 83, 1933.
KAY, H. D. Physiol. Rev. **12**: 384, 1932.
KLEIN, H. J. Dental Res. **9**: 5, 1929.
KLINKE, K. Ergebn. d. Physiol. **26**: 235, 1928.
LEITCH, I. Nutrition Abstr. and Rev. **2**: 451, 1933.
MACCALLUM, W. G. Medicine **3**: 137, 1924.
MARSHALL, J. A. Pacific Dental Gaz. **40**: 243, 1932.
Dental Cosmos **75**: 773, 1933.
MELLANBY, M. Brit. Med. J. **2**: 749, 1932.
Medical Research Council. Special Rept. Series no. 191 (1934).
ORR, J. B. Minerals and pastures. London, 1929.
J. Soc. Chem. Ind. **44**: 964, 1925.
PETERS, J. P. AND D. D. VAN SLYKE. Quantitative clinical chemistry. I. Interpretations. 1932.
ROBISON, G. The significance of phosphoric esters in metabolism. Herter Lectures, 1932.
SHEAR, M. J. Am. J. Cancer **18**: 924, 1933.
SHOHL, A. T. Ann. Rev. Biochem. **2**: 207, 1933.
STEWART, C. P. AND G. H. PERCIVAL. Physiol. Rev. **8**: 283, 1928.
THOMSON, D. L. AND J. B. COLLIP. Ann. Rev. Biochem. **1**: 413, 1932; **2**: 231, 1933.
Physiol. Rev. **12**: 309, 1932.
ZWARENSTEIN, H. Biol. Rev. **9**: 299, 1934.

REFERENCES TO ORIGINAL ARTICLES

ABDERHALDEN, E. Ztschr. physiol. Chem. **25**: 65, 1898.
ADOLPH, W. H. AND S. CHEN. J. Nutr. **5**: 379, 1932.
ALBRIGHT, F., W. BAUER, M. ROPES AND J. C. AUB. J. Clin. Invest. **7**: 139, 1929.
ALBRIGHT, F., W. BAUER AND J. C. AUB. J. Clin. Invest. **10**: 187, 1931.
ALBRIGHT, F., W. BAUER, J. R. COCKRILL AND R. ELLSWORTH. J. Clin. Invest. **9**: 659, 1931.
ALLARDYCE, W. J. Am. Journ. Physiol. **98**: 417, 1931.
ANDERSON, D. F. Brit. J. Exp. Path. **14**: 155, 1933.
ANDERSON, G. H. Biochem. J. **17**: 43, 1923.
ANDREYEV, L., AND L. I. PUGSLEY. J. Physiol. **80**: 96, 1933.
ARNOLD, R. M. AND L. B. MENDEL. J. Biol. Chem. **72**: 189, 1927.
ASHER, D. W. AND J. J. JONES. J. Biol. Chem. **100**: 333, 343, 1931.

ASHFORD, C. A. Biochem. J. **24**: 661, 1930.
AUB, J. C., W. BAUER, C. HEATH AND M. ROPES. J. Clin. Invest. **7**: 97, 1929.
AUB, J. C., F. ALBRIGHT, W. BAUER AND E. ROSSMEISL. J. Clin. Invest. **11**: 211, 1932.
BAKWIN, H. AND R. M. BAKWIN. Am. J. Hyg. **15**: 766, 1932.
BÄR, G. F. J. M. Endokrinol. **1**: 421, 1928.
BARBOUR, H. G. AND W. F. TAYLOR. J. Pharm. Exp. Therap. **42**: 321, 1931.
BARBOUR, H. G. AND J. E. WINTER. J. Pharm. Exp. Therap. **35**: 425, 1929.
J. Pharm. Exp. Therap. **43**: 607, 1931.
BARKUS, O. O. Am. J. Physiol. **69**: 35, 1924.
BARRIO, N. G. J. Lab. Clin. Med. **9**: 54, 1923–24.
BASSETT, S. H., C. A. ELDEN AND W. S. MCCANN. J. Nutr. **5**: 1, 1932.
BAUDOUIN, A., J. LEWIN AND E. AZÉRAD. Compt. rend. soc. biol. **109**: 1265, 1932.
Compt. rend. soc. biol. **112**: 367, 1933.
BAUER, W. AND M. ROPES. J. A. M. A. **87**: 1902, 1926.
BAUER, W., F. ALBRIGHT AND J. C. AUB. J. Clin. Invest. **7**: 75, 1929.
BAUER, W., J. C. AUB AND F. ALBRIGHT. J. Exp. Med. **49**: 145, 1929.
BAXTER, H. J. Biol. Chem. **102**: 203, 1933.
BECHER, E. Klin. Wchnschr. **16**: 202, 1932.
BĚCKA, J. Ztschr. ges. exp. Med. **67**: 252, 1929.
BECKER, R. B., C. H. ECKLES AND L. S. PALMER. J. Dairy Sci. **10**: 169, 1927.
BECKS, H. Proc. Soc. Exp. Biol. Med. **26**: 93, 1928.
BENDIEN, W. M. AND I. SNAPPER. Biochem. Ztschr. **260**: 105, 1933.
BENEDICT, E. M. AND K. B. TURNER. J. Clin. Invest. **9**: 263, 1930.
BENJAMIN, H. R. AND A. F. HESS. J. Biol. Chem. **100**: 27, 1933.
BENJAMIN, H. R. J. Biol. Chem. **100**: 57, 1933.
BENJAMIN, H. R., A. F. HESS AND J. GROSS. J. Biol. Chem. **103**: 383, 1933.
BENJAMIN, H. R. AND A. F. HESS. J. Biol. Chem. **103**: 629, 1933.
BERG, B. N., A. F. HESS AND E. SHERMAN. J. Exp. Med. **47**: 105, 1928.
BERGEIM, O. J. Biol. Chem. **70**: 51, 1926.
Arch. Int. Med. **37**: 110, 1926.
BERNARDI, A. AND M. A. SCHWARZ. Biochem. Ztschr. **262**: 380, 387, 1933.
BERNHARD, A. AND J. J. BEAVER. J. Biol. Chem. **69**: 113, 1926.
BETHKE, R. M., C. H. KICK AND W. WILDER. J. Biol. Chem. **98**: 389, 1932.
BETHKE, R. M., H. STEENBOCK AND M. T. NELSON. J. Biol. Chem. **58**: 71, 1923–24.
BEZNÁK, A. VON. Biochem. Ztschr. **225**: 305, 1930.
Biochem. Ztschr. **225**: 295, 1930.
Pflüger's Arch. **228**: 604, 1931.
BILLS, C. E. J. Biol. Chem. **72**: 751, 1927.
BILLS, C. E., E. M. HONEYWELL, A. M. WIRICK AND M. NUSSMEIER. J. Biol. Chem. **90**: 619, 1931.
BINGER, C. A. L. J. Pharm. Exp. Therap. **10**: 105, 1917.
BISCHOFF, G. Ztschr. physiol. Chem. **188**: 247, 1930.
BLATHERWICK, N. R. AND M. L. LONG. J. Biol. Chem. **52**: 125, 1922.
BLOOM, C. J. Am. Med., Special Section on General Practice **39**: 6, 22, 1933.
BLOOR, W. R. J. Biol. Chem. **23**: 317, 1915.
J. Biol. Chem. **24**: 447, 1915.

BOGERT, L. J. AND E. E. KIRKPATRICK. J. Biol. Chem. **54:** 375, 1922.
BOGERT, L. J. AND E. J. MCKITTRICK. J. Biol. Chem. **54:** 363, 1922.
BOGERT, L. J. AND A. B. HASTINGS. J. Biol. Chem. **94:** 473, 1931–32.
BOGERT, L. J. AND R. K. TRAIL. J. Biol. Chem. **54:** 387, 1922.
BOGERT, L. J. AND E. D. PLASS. J. Biol. Chem. **56:** 297, 1923.
J. Biol. Chem. **56:** 309, 1923.
BOLLIGER, A. AND I. HARTMAN. J. Biol. Chem. **64:** 91, 1925.
BOLLIGER, A. J. Biol. Chem. **69:** 721, 1926.
BOMSKOV, C. AND E. KRIEGER. Ztschr. Kinderheilk. **52:** 47, 1931.
BOMSKOV, C. AND G. MOSCHINSKI. Ztschr. ges. exp. Med. **83:** 326, 1932.
BOMSKOV, C. Ztschr. physiol. Chem. **210:** 67, 1932.
BOMSKOV, C. AND G. SEEMANN. Ztschr. ges. exp. Med. **89:** 771, 1933.
BOYD, G. L., A. M. COURTNEY AND I. F. MACLACHLAN. Am. J. Dis. Child. **32:** 29, 1926.
BOYD, J. D., C. L. DRAIN AND G. STEARNS. J. Biol. Chem. **103:** 327, 1933.
BRAIN, R. T., H. D. KAY AND P. G. MARSHALL. Biochem. J. **22:** 628, 1928.
BRAND, T., F. HOLTZ AND W. PUTSCHAR. Arch. exp. Path. Pharm. **167:** 113, 1932.
BRAND, T. VON AND F. HOLTZ. Ztschr. physiol. Chem. **185:** 217, 1929.
BREDIG, M. A. Ztschr. physiol. Chem. **216:** 239, 1933.
BRIGGS, A. P. Arch. Int. Med. **37:** 440, 1926.
BRINKMAN, R. AND E. VAN DAM. Kon. Akad. van Wetenschappen, Proc. Sec. Sciences **22:** 762, 1920.
BROOKE, R. O. AND A. H. SMITH. J. Biol. Chem. **100:** 105, 1933.
BROOKFIELD, R. W. Biochem. J. **27:** 173, 1933.
BROUGHER, J. C. Am. J. Physiol. **80:** 411, 1927.
Am. J. Physiol. **87:** 221, 1928.
BROWN, H. J. Biol. Chem. **68:** 729, 1926.
J. Biol. Chem. **75:** 789, 1927.
BROWN, H. AND S. G. RAMSDELL. J. Exp. Med. **49:** 705, 1929.
BROWN, H. B. AND A. T. SHOHL. J. Biol. Chem. **86:** 245, 1930.
BROWN, H. B., A. T. SHOHL, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN. J. Biol. Chem. **98:** 207, 1932.
BROWN, W. H. J. Exp. Med. **47:** 539, 563, 637, 1928.
BRULL, L. Arch. Internat. Physiol. **32:** 138, 1930.
BUCKNER, G. D. AND J. H. MARTIN. J. Biol. Chem. **41:** 195, 1920.
BUCKNER, G. D., J. H. MARTIN AND F. E. HULL. Am. J. Physiol. **93:** 86, 1930.
BUCKNER, G. D., J. H. MARTIN AND W. M. INSKO. Am. J. Physiol. **94:** 692, 1930.
BUCKNER, G. D., J. H. MARTIN AND A. M. PETER. Bull. no. 252, Kentucky Agric. Exp. Station, 1924.
Am. J. Physiol. **72:** 253, 1925.
BUCKNER, G. D. AND A. M. PETER. J. Biol. Chem. **54:** 5, 1922.
BULBRING, E. Arch. Exp. Path. Pharm. **162:** 209, 1931.
BULGER, H. A. AND F. GAUSMANN. J. Clin. Invest. **12:** 1135, 1933.
BULGER, H. A., J. P. PETERS, A. J. EISENMAN AND C. LEE. J. Clin. Invest. **2:** 213, 1926.
BULGER, H. A., H. H. DIXON, D. P. BARR AND O. SCHREGARDUS. J. Clin. Invest. **9:** 143, 1930.
BUNTING, R. W. J. Am. Dent. Assoc. **11:** 64, 1924.

BURNS, C. M. Biochem. J. **27**: 22, 1933.
BYROM, F. B. AND H. D. KAY. Brit. J. Exp. Path. **8**: 429, 1927.
BYROM, F. B. Brit. J. Exp. Path. **10**: 10, 1929.
CAJORI, F. A., C. Y. CROUTER AND R. PEMBERTON. Arch. Int. Med. **37**: 92, 1926.
CALLISON, E. W., J. LANDER AND F. P. UNDERHILL. J. Pharm. Exp. Therap. **38**: 385, 1930.
CAMERON, A. T. AND V. H. K. MOORHOUSE. J. Biol. Chem. **63**: 687, 1925.
CANNAVÓ, L. AND R. INDOVINA. Biochem. Ztschr. **261**: 45, 1932.
CANNAVÓ, L. Biochem. Ztschr. **245**: 234, 1932.
CANTAROW, A. Arch. Int. Med. **44**: 670, 1929.
Arch. Int. Med. **44**: 834, 1929.
CARSWELL, H. E. AND J. E. WINTER. J. Biol. Chem. **93**: 411, 1931.
CAVINS, A. W. J. Biol. Chem. **59**: 237, 1924.
CHARLES, E. AND L. HOGBEN. Quart. J. Exp. Physiol. **23**: 343, 1933.
CHUTE, A. L. AND L. IRVING. Trans. Roy. Soc. Canada **26**: 141, 1932.
CLARK, G. W. Univ. Calif. Pub. Physiol. **5**: 195, 1926.
COCKRILL, J. R. Arch. Neurol. and Psychist. **25**: 1297, 1927.
COHEN, H. Quart. J. Med. **17**: 289, 1923–24.
CHEYMOL, J. AND A. QUINQUAUD. Bull. Soc. chim. biol. **14**: 1044, 1046, 1048, 1932.
COLE, V. V. AND F. C. KOCH. J. Biol. Chem. **94**: 263, 1932.
COMMON, R. H. J. Agric. Sci. **22**: 577, 1932.
COONS, C. M. AND K. BLUNT. J. Biol. Chem. **86**: 1, 1930.
CORI, C. F. AND H. L. GOLTZ. Am. J. Physiol. **72**: 256, 1925.
CORRELL, J. T. AND J. S. HUGHES. J. Biol. Chem. **103**: 511, 1933.
CORTEN, M. H. AND I. ESTERMANN. Ztschr. physik. Chem. **136**: 228, 1928.
CORTEN, M. H. Cent. Allg. Path. u. Path. Anat. **44**: 144, 1928.
COX, W. M., JR. AND M. IMBODEN. Proc. Amer. Soc. Biol. Chem., p. xviii, March, 1934.
CSAPÓ, J. AND J. FAUBL. Biochem. Ztschr. **150**: 509, 1924.
CULHANE, K. Biochem. J. **21**: 1015, 1927.
CULLEN, G. E., W. E. WILKINS AND T. R. HARRISON. J. Biol. Chem. **102**: 403, 1933.
CRUICKSHANK, E. W. H. Biochem. Journ. **17**: 13, 1923.
CUSHNY, A. R. J. Physiol. **53**: 391, 1920.
DAILY, M. E. J. Biol. Chem. **93**: 5, 1931.
DALE, H., A. MARBLE AND H. MARKS. Proc. Roy. Soc. **111B**: 522, 1932.
DAVENPORT, H. A., H. H. DIXON AND S. W. RANSON. J. Biol. Chem. **83**: 741, 1921.
DENIS, W. AND R. C. CORLEY. J. Biol. Chem. **65**: 609, 1925.
DEVANEY, G. M., H. E. MUNSELL AND H. W. TITUS. Poult. Sci. **12**: 215, 1933.
DILLMAN, L. M. AND M. B. VISSCHER. J. Biol. Chem. **103**: 791, 1933.
Director, Vet. Services, Animal Ind. Union, South Africa, Mineral Metabolism XVIII. 18, part 2, 611–30 (1932).
DIXON, H. H., H. A. DAVENPORT AND S. W. RANSON. J. Biol. Chem. **83**: 737, 1929.
DRINKER, C. K. AND M. E. FIELD. Am. J. Physiol. **97**: 32, 1931.
DRUKER, P. AND F. FABER. J. Biol. Chem. **68**: 57, 1926.
DUKE-ELDER, W. S. J. Physiol. **68**: 155, 1929.

DUPRE, E. F. AND E. SEMEONOFF. J. Biol. Chem. **94**: 341, 1932.
ECKLES, C. H., T. W. GULLICKSON AND L. S. PALMER. Technical Bulletin no. 91, Univ. Minn. Agric. Expt. Station, 1932.
EICHHOLTZ, F. AND R. BERG. Biochem. Ztschr. **225**: 352, 1930.
ELIAS, H. AND F. KORNFELD. Klin. Wchnschr. **2**: 1206, 1923.
ELLSWORTH, R. J. Clin. Invest. **8**: 139, 1930.
J. Clin. Invest. **11**: 1011, 1932.
ELMSLIE, W. P. AND H. STEENBOCK. J. Biol. Chem. **82**: 611, 1929.
ELVEHJEM, C. A. AND B. E. KLINE. J. Biol. Chem. **103**: 733, 1933.
ENDER, F. Ztschr. Vitaminforschung **2**: 241, 1933.
EULER, H. VON AND M. RYDBLOM. Biochem. Ztschr. **241**: 14, 1931.
EULER, H. VON AND E. VIRGIN. Biochem. Ztschr. **249**: 393, 1932.
FAIRHALL, L. T. Am. J. Physiol. **84**: 378, 1928.
FARQUHARSON, R. F., W. T. SALTER, D. M. TIBBETTS AND J. C. AUB. J. Clin. Invest. **10**: 221, 1931.
FARQUHARSON, R. F., W. T. SALTER AND J. C. AUB. J. Clin. Invest. **10**: 251, 1931.
FARQUHARSON, R. F. AND D. M. TIBBETTS. J. Clin. Invest. **10**: 271, 1931.
FIELD, M. E. AND C. K. DRINKER. Am. J. Physiol. **97**: 40, 1931.
FIELD, M. E. AND C. K. DRINKER. Am. J. Physiol. **98**: 66, 1931.
FISCHER, H. AND A. HENDSCHEL. Ztschr. physiol. Chem. **198**: 33, 1931.
FISKE, C. H. J. Biol. Chem. **49**: 171, 1921.
FISKE, C. H. AND M. A. LOGAN. J. Biol. Chem. **93**: 211, 1931.
FITCH, C. P., W. L. BOYD, C. H. ECKLES, T. W. GULLICKSON, L. S. PALMER AND C. KENNEDY. Cornell Veterinarian **22**: 156, 1932.
FORBES, E. B., F. M. BEEGLE, C. M. FRITZ, L. E. MORGAN AND S. N. RHUE. Bull. Ohio Agric. Expt. Station, no. 295, p. 323, 1916. First paper.
Bull. Ohio Agric. Expt. Station, no. 308, p. 451, 1917.
FORBES, E. B., J. O. HALVERSON, L. E. MORGAN, J. A. SCHULZ, C. E. MANGELS, S. N. RHUE AND G. W. BURKE. Bull. Ohio Agric. Expt. Station, no. 330, p. 91, 1918.
FORBES, E. B., J. O. HALVERSON, L. E. MORGAN AND J. A. SCHULZ. Ohio Agric. Expt. Station Bull. no. 347, p. 3, 1921.
FORBES, E. B., J. A. SCHULZ, C. H. HUNT, A. R. WINTER AND R. F. REMLER. J. Biol. Chem. **52**: 281, 1922.
FOSBINDER, R. I. J. Amer. Chem. Soc. **51**: 1345, 1929.
FRASER, N. H. H. Biochem. J. **26**: 2166, 1932.
FREMONT-SMITH, F. Arch. Neurol. and Psychiat. **17**: 317, 1927.
FREMONT-SMITH, F., M. E. DAILY, H. H. MERRITT, M. P. CARROLL AND G. W. THOMAS. Arch. Neurol. and Psychiat. **25**: 1271, 1931.
GARGILL, S. L., D. ROURKE AND H. L. BLUMGART. Arch. Int. Med. **45**: 879, 1930.
GILLIGAN, D. R., M. C. VOLK AND M. D. ALTSCHULE. J. Biol. Chem. **103**: 745, 1933.
GIVENS, M. H. AND L. B. MENDEL. J. Biol. Chem. **31**: 421, 1917.
GOLDBLATT, H. Biochem. J. **18**: 414, 1924.
GOSS, H. AND C. L. A. SCHMIDT. J. Biol. Chem. **86**: 417, 1930.
GOUGH, J., J. B. DUGUID AND D. R. DAVIES. Brit. J. Exp. Path. **14**: 137, 1933.
GRANT, S. B. AND A. GOLDMAN. Am. J. Physiol. **52**: 209, 1920.

GREENBERG, D. M. J. Biol. Chem. **79**: 177, 1928.
Proc. Soc. Exp. Biol. and Med. **27**: 514, 1930.
Proc. Soc. Exp. Biol. and Med. **29**: 721, 1932.
Proc. Soc. Exp. Biol. and Med. **30**: 1005, 1933.
GREENBERG, D. M. AND M. GREENBERG. J. Biol. Chem. **94**: 373, 1931.
GREENBERG, D. M. AND L. D. GREENBERG. J. Biol. Chem. **99**: 1, 1932.
GREENBERG, D. M. AND L. GUNTHER. J. Biol. Chem. **85**: 491, 1930.
Arch. Int. Med. **46**: 72, 1930.
Arch. Int. Med. **50**: 855, 1932.
GREENBERG, D. M., S. P. LUCIA, M. A. MACKEY AND E. V. TUFTS. J. Biol. Chem. **100**: 139, 1933.
GREENBERG, D. M. AND M. A. MACKEY. J. Biol. Chem. **98**: 265, 1932.
J. Biol. Chem. **96**: 419, 1932.
GREENBERG, D. M. AND C. L. A. SCHMIDT. J. Gen. Physiol. **8**: 271, 1926.
GREENE, C. H. AND M. H. POWERS. J. Biol. Chem. **91**: 183, 1931.
GREENE, C. H., J. L. BOLLMAN, N. M. KEITH AND E. G. WAKEFIELD. J. Biol. Chem. **91**: 203, 1931.
GREENWALD, I. J. Biol. Chem. **14**: 369, 1913.
J. Pharm. Exp. Therap. **11**: 281, 1918.
GREENWALD, I. AND J. GROSS. J. Biol. Chem. **66**: 185, 1925.
GREENWALD, I. J. Biol. Chem. **67**: 1, 1926.
GREENWALD, I. AND J. GROSS. J. Biol. Chem. **68**: 325, 1926.
GREENWALD, I. J. Biol. Chem. **93**: 551, 1931.
GREISHEIMER, E. M., O. H. JOHNSON AND M. RYAN. Am. J. Med. Sci. **177**: 704, 1929.
GREVILLE, G. D. Biochem. J., **25**: 1931, 1931.
GROLLMAN, A. J. Biol. Chem. **72**: 565, 1927.
GUEST, G. M. AND J. WARKANY. J. Biol. Chem. **100**: 445, 1934.
GUILBERT, H. R. AND G. H. HART. Hilgardia **5**: 101, 1930.
GULÁCSY, Z. Biochem. Ztschr. **251**: 162, 1932.
GUNTHER, L. AND D. M. GREENBERG. Arch. Int. Med. **45**: 983, 1930.
Arch. Int. Med. **45**: 67, 1930.
Arch. Int. Med. **47**: 660, 1931.
GÜRSCHING, J. AND H. KRAUT. Arch. Exp. Path. Pharm. **167**: 146, 1932.
GUYATT, B. L., H. D. KAY AND H. D. BRANION. J. Nutr. **6**: 313, 1933.
HAAG, J. R. AND L. S. PALMER. J. Biol. Chem. **76**: 367, 1928.
HAM, A. W. Arch. Path. **14**: 613, 1932.
HAM, A. AND B. C. PORTUONDO. Arch. Path. **16**: 1, 1933.
HAMMETT, F. S. J. Biol. Chem. **57**: 285, 1925.
J. Biol. Chem. **72**: 527, 1927.
HANDELMAN, I., A. ROSE AND C. P. SHERWIN. Arch. Int. Med. **37**: 725, 1926.
HARNES, A. F. J. Exp. Med. **48**: 549, 1928.
J. Exp. Med. **49**: 287, 1929.
HARRIS, L. J. Lancet **222**: 1031, 1932.
HARRIS, L. J. AND J. R. M. INNES. Biochem. J. **25**: 367, 1931.
HARRIS, L. J. AND T. MOORE. Biochem. J. **22**: 1461, 1928.
Biochem. J. **23**: 261, 1929.
HARROP, G. A. AND E. M. BENEDICT. J. Biol. Chem. **59**: 683, 1924.

HART, E. B., H. STEENBOCK AND C. A. HOPPERT. J. Biol. Chem. **48**: 33, 1921.
HART, E. B., H. STEENBOCK, C. A. HOPPERT AND G. C. HUMPHREY. J. Biol. Chem. **53**: 21, 1922.
HART, E. B., H. STEENBOCK, C. A. HOPPERT, R. M. BETHKE AND G. C. HUMPHREY. J. Biol. Chem. **54**: 75, 1922.
HART, E. B., H. STEENBOCK, C. A. HOPPERT AND G. C. HUMPHREY. J. Biol. Chem. **58**: 43, 1923.
HART, E. B., H. STEENBOCK, C. A. ELVEHJEM, H. SCOTT AND J. C. HUMPHREY. J. Biol. Chem. **67**: 371, 1926.
HART, E. B., H. STEENBOCK, H. SCOTT AND G. C. HUMPHREY. J. Biol. Chem. **71**: 263, 1927.
HART, E. B., H. STEENBOCK, S. W. KLETZIEN AND H. SCOTT. J. Biol. Chem. **71**: 271, 1927.
HART, E. B., H. STEENBOCK AND H. SCOTT. J. Biol. Chem. **73**: 59, 1927.
HART, E. B., H. STEENBOCK, E. C. TEUT AND G. C. HUMPHREY. J. Biol. Chem. **84**: 359, 1929. J. Biol. Chem. **84**: 367, 1929.
HART, E. B., H. STEENBOCK, O. L. KLINE AND G. C. HUMPHREY. J. Biol. Chem. **86**: 145, 1930.
J. Dairy Sci. **14**: 307, 1931.
HART, E. B., H. STEENBOCK, S. LEPKOVSKY, S. W. F. KLETZIEN, J. G. HALPIN AND O. N. JOHNSON. J. Biol. Chem. **65**: 579, 1925.
HART, G. H., H. R. GUILBERT AND H. GOSS. Bull. no. 543, Univ. California Coll. Agric., 1932.
HART, M. C., D. TOURTELLOTTE AND F. W. HEYL. J. Biol. Chem. **76**: 143, 1928.
HARTMANN, A. F., D. C. DARROW AND M. MORTON. J. Clin. Invest. **6**: 127, 1928.
HASTINGS, A. B. AND C. B. HUGGINS. Proc. Soc. Exp. Biol. Med. **30**: 458, 1933.
HASTINGS, A. B., C. D. MURRAY AND J. SENDROY. J. Biol. Chem. **71**: 723, 1927.
HAUCK, H. M., H. STEENBOCK AND H. T. PARSONS. Am. J. Physiol. **103**: 480, 1933.
HAURY, V. G. J. Biol. Chem. **89**: 467, 1930.
Am. J. Physiol. **103**: 489, 1933.
HAYNES, F. W. Am. J. Physiol. **101**: 223, 612, 1932.
Am. J. Physiol. **101**: 232, 1932.
HERBERT, F. K. Biochem. J. **27**: 1978, 1933.
HERTZ, W. Biochem. Ztschr. **217**: 337, 1930.
HERZFELD, E., H. LUBOWSKI AND R. KRÜGER. Folia Haemat. **41**: 73, 1930.
HESS, A. F. AND H. R. BENJAMIN. Proc. Soc. Exp. Biol. Med. **30**: 1358, 1933.
HESS, A. F., H. R. BENJAMIN AND J. GROSS. J. Biol. Chem. **94**: 1, 1931.
HESS, A. F., B. N. BERG AND E. SHERMAN. J. Exp. Med. **47**: 115, 1928.
HESS, A. F., F. S. BERLINER AND M. WEINSTOCK. J. Biol. Chem. **94**: 9, 1931.
HESS, A. F., J. GROSS, M. WEINSTOCK AND F. S. BERLINER. J. Biol. Chem. **98**: 625, 1932.
HESS, A. F. AND J. M. LEWIS. J. A. M. A. **91**: 703, 1928.
J. A. M. A. **99**: 647, 1932.
HESS, A. F., J. M. LEWIS AND B. ROMAN. Dental Cosmos **74**: 1053, 1932.
HESS, A. F. AND M. WEINSTOCK. Am. J. Dis. Child. **34**: 845, 1927.
HESS, A. F., M. WEINSTOCK, H. R. BENJAMIN AND J. GROSS. J. Biol. Chem. **90**: 737, 1931.

HESS, A. F., M. WEINSTOCK AND J. GROSS. Proc. Soc. Exp. Biol. Med. **30**: 1357, 1933.
HESS, J. H., J. K. CALVIN, C. C. WANG AND A. FELCHER. Am. J. Dis. Child. **26**: 271, 1923.
HETÉNYI, G. AND A. VON GAÁL. Ztschr. ges. exp. Med. **75**: 516, 1931.
HEUBNER, W. AND P. RONA. Biochem. Ztschr. **93**: 187, 1919.
Biochem. Ztschr. **135**: 248, 1923.
HIGGINS, G. M. AND C. SHEARD. Am. J. Physiol. **85**: 299, 1928.
HIRSCHFELDER, A. D. J. Pharm. Exp. Therap. **48**: 277, 1933.
HIRSCHFELDER, A. D. AND E. R. SERLES. J. Pharm. Exp. Therap. **29**: 441, 1926.
HJORT, A. M. J. Biol. Chem. **65**: 783, 1925.
HOGBEN, L. AND E. CHARLES. J. Exp. Biol. **9**: 139, 1932.
HOLE, J. C. J. Pharm. Exp. Therap. **32**: 309, 1927.
HOLT, L. E. J. Biol. Chem. **66**: 23, 1925.
HOLT, L. E., V. K. LA MER AND H. B. CHOWN.
J. Biol. Chem. **64**: 509, 567, 1925.
J. Biol. Chem. **64**: 579, 1925.
HOLTZ, F. AND E. SCHREIBER. Ztschr. physiol. Chem. **191**: 1, 1930.
HOMMERBERG, C. Biochem. Ztschr. **258**: 154, 1932.
HOOBLER, B. R. Am. J. Dis. Child. **2**: 107, 1911.
HOWE, P. R. J. Dent. Res. **7**: 145, 1927.
J. Am. Dent. Assn. **11**: 1149, 1924.
HOWLAND, J. AND B. KRAMER. Am. J. Dis. Child. **22**: 105, 1921.
HOWLAND, J., W. K. MARRIOTT AND B. KRAMER. J. Biol. Chem. **68**: 721, 1926.
HUBBELL, R. B. AND R. W. BUNTING. J. Nutr. **5**: 599, 1932.
HUFFMAN, C. F., C. S. ROBINSON, O. B. WINTER AND R. E. LARSON. J. Nutr., **2**: 471, 1930.
HUGHES, J. S., W. L. LATSHAW AND B. L. SMITS. J. Biol. Chem. **74**: 30, 1927. See also Science **65**: 264, 1927.
HUGHES, T. A. AND D. L. SHRIVASTAVA. Indian J. Med. Res. **17**: 461, 1929.
HUGHES, T. A., D. L. SHRIVASTAVA AND P. M. SAHAI. Indian J. Med. Res. **17**: 470, 1929.
HUNSCHER, H. A. J. Biol. Chem. **86**: 37, 1930.
HUNSCHER, H. A., F. COPE, A. NOLL AND I. G. MACY. Proc. Amer. Soc. Biol. Chem. **8**: LV, 1933.
HUNTER, D. AND J. C. AUB. Quart. J. Med. **20**: 123, 1926.
HUNTER, J. E., R. A. DUTCHER AND H. C. KNANDEL. Proc. Soc. Exp. Biol. Med. **31**: 70, 1933.
INGRAHAM, R. C., C. LOMBARD AND M. B. VISSCHER. J. Gen. Physiol. **16**: 637, 1933.
IRVING, L. J. Biol. Chem. **68**: 513, 1926.
IRVING, L. AND A. L. CHUTE. J. Cell. Comp. Physiol. **2**: 157, 1932.
JACOBSON, S. A. Arch. Path. **15**: 18, 1933.
JACOBY, M. AND H. FRIEDEL. Biochem. Ztschr. **260**: 451, 1933.
JAFFE, H. L. Arch. Path. **15**: 83, 1933.
JAFFE, H. L., A. BODANSKY AND J. P. CHANDLER. J. Exp. Med. **56**: 823, 1932.
JEANS, P. C. AND G. STEARNS. Am. J. Dis. Child. **42**: 708, 1931.
JENNER, H. D. AND H. D. KAY. J. Biol. Chem. **93**: 733, 1931.

JODLBAUER, A. Arch. exp. Path. Pharm. **164:** 464, 1932.
JOHNSTON, C. G., I. S. RAVDIN, J. H. AUSTIN AND J. L. MORRISON. Am. J. Physiol. **99:** 648, 1932.
JONES, J. H., M. RAPOPORT AND H. L. HODES. J. Biol. Chem. **86:** 267, 1930.
JONES, J. H. AND G. M. ROBSON. Am. J. Physiol. **103:** 338, 1933.
JONES, M. R. J. Biol. Chem. **49:** 187, 1921.
JUNDELL, I. AND H. MAGNUSSON. Acta. Paediat. **14:** 13, 1932.
KAHN, B. S. AND J. A. ROE. J. A. M. A. **86:** 1761, 1926.
KAPSINOW, R. AND F. P. UNDERHILL. J. Biol. Chem. **82:** 377, 1929.
KARELITZ, S. AND A. T. SHOHL. J. Biol. Chem. **73:** 665, 1927.
KARSHAN, M. J. Dental Res. **13:** 301, 1933.
KARSHAN, M. AND T. ROSEBURG. J. Dental Res. **13:** 305, 1933.
KAY, H. D. AND F. B. BYROM. Brit. J. Exp. Path. **8:** 240, 1927.
KELLY, F. C. AND J. M. HENDERSON. J. Hyg. **29:** 418, 429, 439, 1930.
KENNARD, D. C. Poultry Science **4:** 109, 1925.
KICK, C. H., R. M. BETHKE AND B. H. EDGINGTON. J. Agric. Res. **46:** 1023, 1933.
KIM, M. H. Acta Med. Keijo **12:** 203, 1929.
KING, E. J., H. HULL AND G. E. HALL. Poultry Science **12:** 129, 1933.
KIRK, P. L. AND C. L. A. SCHMIDT. J. Biol. Chem. **76:** 115, 1928.
J. Biol. Chem. **83:** 311, 1929.
KLEINMAN, H. Biochem. Ztschr. **196:** 98, 1928.
KLEMENT, R. Ztschr. physiol. Chem. **184:** 132, 1929.
KLEMENT, R. AND G. TRÖMEL. Ztschr. physiol. Chem. **213:** 263, 1932.
KLETZIEN, S. W. F., V. M. TEMPLIN, H. STEENBOCK AND B. H. THOMAS. J. Biol. Chem. **97:** 265, 1932.
KLINKE, K. Biochem. Ztschr. **213:** 177, 1929.
KORMOS, A. Ztschr. ges. exp. Med. **88:** 513, 1933.
KOTIKOFF, J. A. Jahrb. Kinderheilk. **138:** 280, 1933.
KOZELKA, F. L., E. B. HART AND G. BOHSTEDT. J. Biol. Chem. **100:** 715, 1933.
KRAMER, B. AND J. HOWLAND. J. Nutr. **5:** 39, 1932.
KRAMER, B. AND E. E. TISDALL. J. Biol. Chem. **53:** 241, 1922.
KRAMER, B., F. F. TISDALL AND J. HOWLAND. Am. J. Dis. Child. **22:** 431, 1921.
KRAMER, M. AND M. J. SHEAR. J. Biol. Chem. **79:** 147, 1928.
KREITMAIR, H. AND T. MOLL. Münch. Med. Wchnschr. **75:** 637, 1921.
KRUSE, H. D., E. R. ORENT AND E. V. MCCOLLUM. J. Biol. Chem. **96:** 519, 1932.
J. Biol. Chem. **100:** 603, 1933.
KRUSE, H. D., M. M. SCHMIDT AND E. V. MCCOLLUM. Am. J. Physiol. **105:** 635, 1933.
KYLIN, E. AND G. NYSTROM. Ztschr. ges. exp. Med. **45:** 208, 1925.
LAMELAS, J. Am. J. Physiol. **93:** 111, 1930.
LÁNG, S. AND L. RIGÓ. Arch. exp. Path. Pharm. **139:** 1, 1929.
LASKOWSKI, M. Biochem. Ztschr. **260:** 230, 1933.
Biochem. Ztschr. **265:** 401, 1933.
LAVOLLAY, J. Bull. Soc. Chim. Biol. **13:** 1205, 1931.
LE BLANC, M. AND O. HARNAPP. Ztschr. Elektrochemie **36:** 116, 1930.
LEIBOFF, S. L. J. Biol. Chem. **85:** 758, 1930.
LEITES, S. AND A. KOSLOWA. Arch. exp. Path. Pharm. **169:** 385, 1932.
LIEBERMAN, A. L. J. Pharm. Exp. Therap. **40:** 71, 1930.
J. Pharm. Exp. Therap. **42:** 245, 1931.

LIGHT, R. F., G. MILLER AND C. N. FREY. J. Biol. Chem. **84**: 487, 1929.
LILLY, C. A. J. Nutr. **5**: 175, 1932.
LINDER, G. C. AND C. F. HARRIS. Quart. J. Med. **23**: 195, 1930.
LITTLE, W. L. AND N. C. WRIGHT. Brit. J. Exp. Path. **6**: 129, 1925.
LIU, S. H. Chinese J. Physiol. **1**: 331, 1927.
J. Clin. Invest. **5**: 259, 277, 1927–28.
LOEB, J. Am. J. Physiol. **5**: 362, 1901.
LOEB, R. F. J. Gen. Physiol. **8**: 451, 1925.
LOEB, R. F. AND E. G. NICHOLS. J. Biol. Chem. **74**: 645, 1927.
LOEB, R. F. AND E. G. NICHOLS. J. Biol. Chem. **72**: 687, 1927.
LOEWY, A. AND G. CRONHEIM. Biochem. Ztschr. **256**: 411, 1932.
LUCE, E. M. J. Path. and Bact. **26**: 200, 1923.
MACCALLUM, W. G. AND C. VOEGTLIN. J. Exp. Med. **11**: 118, 1909.
MACOMBER, J. A. J. A. M. A., **88**: 6, 1927.
MACY, I. G. Yale J. Biol. and Med. **4**: 451, 1932.
MACY, I. G., H. A. HUNSCHER, S. S. MCCOSH AND B. NIMS. J. Biol. Chem. **86**: 59, 1930.
MACY, I. G., H. A. HUNSCHER, B. NIMS AND S. S. MCCOSH. J. Biol. Chem. **86**: 17, 1930.
MACY, I. G., B. NIMS, M. BROWN AND H. A. HUNSCHER. Am. J. Dis. Child. **42**: 569, 1931; **43**: 40, 828, 1062, 1932.
MAINZER, F. Ztschr. ges. exp. Med. **56**: 498, 1927.
MARRACK, J. AND G. THACKER. Biochem. J. **20**: 580, 1926.
MARRIOTT, W. M. AND J. HOWLAND. Arch. Int. Med. **18**: 708, 1916.
MARSHALL, J. A. J. Am. Dental Assn. **11**: 6, 1924.
J. Am. Dental Assn. **14**: 3, 1927.
J. Am. Dental Assn. **15**: 295, 1928.
Internat. J. Orthodont., Oral Surg. and Radiol. **17**: 527, 1931.
MARTIN, C. J. AND A. W. PEIRCE. Bull. no. 77, Australia, Council for Sci. and Indust. Res. Melbourne, 1934.
MARTIN, J. P. Quart. J. Med. **19**: 311, 1925.
MASAKI, T. Trans. Japan. Path. Soc. **21**: 693, 1931.
MASSENGALE, O. N. AND M. NUSSMEIER. J. Biol. Chem. **87**: 415, 423, 1930.
MATHEWS, S. A. AND W. C. AUSTIN. Am. J. Physiol. **79**: 708, 1927.
MATHIEU, F. Compt. rend. soc. biol. **113**: 903, 1933.
MATTHEW, C. W. J. Biol. Chem. **74**: 557, 1927.
MATTICK, E. C. V. Biochem. J. **22**: 144, 1928.
MCBAIN, J. H. AND W. L. MCCLATCHIE. J. Am. Chem. Soc. **55**: 1315, 1933.
MCCANCE, R. A. AND E. WATCHOM. Quart. J. Med. **24**: 371, 1931.
MCCANN, G. F. AND M. BARNETT. J. Biol. Chem. **54**: 203, 1922.
MCCLURE, F. J. AND H. H. MITCHELL. J. Biol. Chem. **90**: 297, 1931.
MCCOLLUM, E. V., N. SIMMONDS, H. T. PARSONS, P. G. SHIPLEY AND E. A. PARK. J. Biol. Chem. **45**: 333, 1920.
MCCRUDDEN, F. H. Am. J. Physiol. **17**: 32, 1906.
J. Biol. Chem. **7**: 199, 1909.
MCCULLAGH, E. P. Arch. Int. Med. **42**: 546, 1928.
MCGOWAN, J. P. Biochem. J. **26**: 1138, 1932.
Biochem. J. **27**: 934, 1933.
Biochem. J. **27**: 943, 1933.

McJunkin, F. A., W. R. Tweedy and H. C. Breuhaus. Arch. Path. **14**: 649, 1932.
McLaughlin, L. J. Biol. Chem. **74**: 455, 1927.
McLean, F. G. and A. B. Hastings. Proc. Soc. Exp. Biol. Med. **30**: 1344, 1933.
Medes, G. J. Biol. Chem. **68**: 295, 1926.
Medes, G. and G. J. Humphrey. J. Biol. Chem. **74**: 149, 1927.
Meigs, E. B., N. R. Blatherwick and C. A. Cary. J. Biol. Chem. **37**: 1, 1919.
J. Biol. Chem. **40**: 469, 1919.
Meigs, E. B., W. A. Turner, E. A. Kane and L. A. Shinn. Proc. Am. Soc. Biol. Chem., p. lx, March, 1934.
Mellanby, M. and C. L. Pattison. Brit. Med. J. **1**: 507, 1932.
Meltzer, S. J. and J. Auer. Am. J. Physiol. **21**: 400, 1908.
Mendel, L. B. and S. R. Benedict. Am. J. Physiol. **25**: 1, 25, 1909.
Merritt, H. H. and W. Bauer. J. Biol. Chem. **90**: 215, 233, 1931.
Meyer, zu Hörste, G. Klin. Wchnschr. **11**: 1796, 1932.
Magnesiumrachitis. Monatschr. Kinderheilk. **54**: 203, 1932.
Meysenburg, von L., A. M. Pappenheimer, T. F. Zucker and M. Murray. J. Biol. Chem. **47**: 529, 1921.
Meysenburg, von L. and G. F. McCann. J. Biol. Chem. **47**: 541, 1921.
Michaux, A. Compt. rend. Acad. Sci. **197**: 865, 1933.
Miles, L. M. and C. I. Feng. J. Exp. Med. **41**: 137, 1925.
Miller, H. G. J. Biol. Chem. **67**: 71, 1926.
Miller, H. G., W. W. Yates, R. C. Jones and P. M. Brandt. Am. J. Physiol. **75**: 696, 1925.
Miyamoto, S. and C. L. A. Schmidt. J. Biol. Chem. **99**: 335, 1933.
Möller, H. and G. Trömel. Naturwissenschaften **21**: 346, 1933.
Mond, R. and H. Netter. Pflüger's Arch. **212**: 558, 1926.
Morgan, A. F. and E. A. Garrison. J. Biol. Chem. **85**: 687, 1929.
Morgan, A. F. and J. G. Field. Am. J. Physiol. **105**: 585, 1933.
Morgan, A. F. and E. A. Garrison. Am. J. Physiol. **105**: 596, 1933.
Morgan, A. F., E. A. Garrison and M. J. Hills. Am. J. Physiol. **105**: 608, 1933.
Morgan, A. F., E. A. Garrison, F. Gillum and M. J. Hills. Am. J. Physiol. **105**: 621, 1933.
Morgulis, S. Am. J. Physiol. **84**: 350, 1928.
J. Biol. Chem. **93**: 455, 1931.
Morgulis, S. and A. M. Perley. Am. J. Physiol. **89**: 213, 1929.
J. Biol. Chem. **88**: 169, 1930.
Moritz, A. R. J. Biol. Chem. **66**: 343, 1925.
Morris, N., J. B. Rennie and S. Morris. Brit. J. Exp. Path. **13**: 132, 1932.
Mulder, A. G., I. E. Phillips and M. B. Visscher. J. Biol. Chem. **98**: 269, 1932.
Mussehl, F. E., R. S. Hill, M. J. Blish and C. W. Ackerson. J. Agric. Res. **40**: 191, 1930.
Neal, W. M., L. S. Palmer, C. H. Eckles and T. W. Gullickson. J. Agric. Res. **42**: 115, 1931.
Needles, M. S. and C. M. Marberg. J. Clin. Lab. Med. **18**: 1227, 1933.
Nelson, C. F. and W. E. Burns. J. Biol. Chem. **28**: 237, 1916.
Nelson, C. F. and J. L. Williams. J. Biol. Chem. **28**: 231, 1916.
Neuhausen, B. S. and E. K. Marshall. J. Biol. Chem. **53**: 365, 1922.

NEUHAUSEN, B. S. AND J. B. PINCAS. J. Biol. Chem. **57**: 99, 1923.
NEUWIRTH, I. AND G. B. WALLACE. J. Pharm. Exp. Therap. **35**: 171, 1929.
J. Pharm. Exp. Therap. **45**: 109, 1932.
NICHOLAS, H. O. J. Biol. Chem. **97**: 457, 1932.
NICOLAYSEN, R. Biochem. Ztschr. **248**: 275, 1932.
NITSCHKE, A. Biochem. Ztschr. **192**: 123, 1928.
Klin. Wchnschr. **8**: 794, 1929.
NITZESCU, I. I. Compt. rend. soc. biol. **110**: 1141, 1932.
NORMAN, G. F. J. A. M. A. **102**: 529, 1934.
OBERST, W. F. AND E. D. PLASS. J. Clin. Invest. **11**: 723, 1932.
OKEY, R., J. M. STEWART AND M. L. GREENWOOD. J. Biol. Chem. **87**: 91, 1930.
ORENT, E. R., H. D. KRUSE AND E. V. MCCOLLUM. Am. J. Physiol. **101**: 454, 1932.
ORR, W. J., L. E. HOLT, L. WILKINS AND F. G. BOONE. Am. J. Dis. Child. **26**: 362, 1923, **28**: 574, 1924.
OSBORNE, T. B. AND L. B. MENDEL. J. Biol. Chem. **34**: 131, 1918.
OSNATO, M., J. A. KILLIAN, T. GARCIA AND M. R. MATTICE. Brain **50**: 581, 1927.
OUTHOUSE, J., I. G. MACY AND V. BREKKE. J. Biol. Chem. **78**: 129, 1928.
ÖWRE, A. Med. Rev. **48**: 49, 1931.
PAGE, I. AND J. P. SCOTT. J. Pharm. Exp. Therap. **46**: 431, 1932.
PALMER, L. S. Special Bull. no. 94, Univ. Minnesota, April, 1932.
PALMER, L. S., W. S. CUNNINGHAM AND C. H. ECKLES. J. Dairy Science **13**: 174, 351, 1930.
PALMER, L. S., C. H. ECKLES AND D. J. SHUTTE. Proc. Soc. Exp. Biol. Med. **26**: 58, 1928.
PALMER, L. S., R. A. GORTNER AND R. RUDE. J. Dairy Science **13**: 360, 1930.
PAPPENHEIMER, A. M. Proc. Soc. Exp. Biol. Med. **11**: 504, 1924.
PETERS, J. P. AND L. EISERSON. J. Biol. Chem. **84**: 155, 1929.
PETERS J. P., A. M. WAKEMAN, A. J. EISENMAN AND C. LEE. J. Clin. Invest. **6**: 517, 1929.
PINCUS, J. B. AND B. KRAMER. J. Biol. Chem. **57**: 463, 1923.
PINCUS, J. B., H. A. PETERSON AND B. KRAMER. J. Biol. Chem. **68**: 601, 1926.
PLASS, E. H. AND E. H. TOMKINS. J. Biol. Chem. **56**: 309, 1923.
PORGES, O. AND D. ADLERSBERG. Klin. Wchnschr. **1**: 1200, 1922.
PUGSLEY, L. I. J. Physiol. **76**: 315, 1932.
PUGSLEY, L. I. AND H. SELYE. J. Physiol. **79**: 113, 1933.
QUAGLIARIELLO, G. Accad. dei Lincei, Rome, (classe di scienze fisiche matematiche e naturali) series 6, volume 9, p. 1029, 1929.
RAVDIN, I. S., C. G. JOHNSTON, C. RIEGEL AND S. L. WRIGHT, JR. Am. J. Physiol. **100**: 317, 1932.
RAVDIN, I. S., C. RIEGEL AND J. L. MORRISON. Ann. Surg. **91**: 801, 1930.
REED, C. I. J. Biol. Chem. **77**: 547, 1928.
REED, C. I., L. M. DILLMAN, A. A. THACKER AND R. I. KLEIN. J. Nutr. **6**: 371, 1933.
REICHE, F. Med. Klinik. **23**: 1820, 1927.
REWBRIDGE, A. G. AND E. ANDREWS. Proc. Soc. Exp. Biol. Med. **28**: 126, 1930.
RIDDLE, O. AND J. KŘÍŽENECKÝ. Am. J. Physiol. **97**: 343, 1931.
RIESENFELD, E. A., I. HANDELMAN AND A. R. ROSE. Am. J. Dis. Child. **30**: 646, 1925.

ROBINSON, C. S., C. F. HUFFMAN AND M. F. MASON. J. Biol. Chem. **84:** 256, 1929.
RONA, P. AND D. TAKAHASHI. Biochem. Ztschr. **31:** 336, 1911.
Biochem. Ztschr. **49:** 370, 1913.
ROSE, M. S. J. Biol. Chem. **41:** 349, 1920.
ROSE, M. S. AND G. MACLEOD. J. Biol. Chem. **57:** 305, 1923.
RUSSELL, W. C. AND F. G. MCDONALD. J. Biol. Chem. **84:** 463, 1929.
RUSSELL, W. C., M. W. TAYLOR AND D. E. WILCOX. J. Biol. Chem. **99:** 109, 1932.
RYMER, M. R. AND R. C. LEWIS. J. Biol. Chem. **95:** 441, 1932.
SALIT, P. W. Arch. Ophthal. **4:** 379, 1930.
J. Biol. Chem. **104:** 275, 1934.
SALVESEN, H. A. Acta Med. Scandinav. Suplementum 6 (1923–24).
Acta Med. Scandinav. **69:** 126, 1928.
SALVESEN, H. A. AND G. C. LINDER. J. Biol. Chem. **58:** 617, 1924.
J. Biol. Chem. **58:** 635, 1924.
SANDERS, G. P. J. Biol. Chem. **30:** 747, 1931.
SARTORY, A., R. SARTORY, J. MEYER AND E. KELLER. Compt. rend. Acad. Sci. **195:** 400, 1932.
SCHEPETINSKY, A. AND M. KAFITIN. Arch. Gynäkol. **136:** 379, 1929.
SCHMIDT, C. L. A. AND D. R. HOAGLAND. Univ. Calif. Pub. Path. **2:** 215, 1919.
SCHMITZ, H. W., E. L. ROHDENBERG AND V. C. MYERS. Arch. Int. Med. **37:** 233, 1926.
SCHOLTZ, H. G. Biochem. Ztschr. **231:** 135, 1931.
Biochem. Ztschr. **249:** 384, 1933.
SCHÖNBERGER, S. Biochem. Ztschr. **251:** 167, 1932.
SCHULTEN, H. Biochem. Ztschr. **164:** 47, 1925.
SCRIVER, W. DE M. AND E. M. VENNING. J. Clin. Invest. **13:** 139, 1934.
SEARLE, O. M. AND J. J. MICHAELS. Am. J. Physiol., **103:** 455, 1933.
SELYE, H. Arch. Path. **14:** 60, 1932.
Endocrinol. **16:** 547, 1932.
SEMSROTH, K. AND H. B. MCCLUGAGE. Arch. Path. **14:** 79, 1932.
SENDROY, J. AND A. B. HASTINGS. J. Biol. Chem. **71:** 783, 797, 1927.
SHAPIRO, H. A. AND H. ZWARENSTEIN. J. Exp. Biol. **10:** 186, 1933.
SHEAR, M. J. AND M. M. OFFNER. J. Biol. Chem. **91:** 291, 1931.
SHEAR, M. J. AND B. KRAMER. J. Biol. Chem. **79:** 105, 121, 125, 1928.
SHEAR, M. J., M. WASHBURN AND B. KRAMER. J. Biol. Chem. **83:** 697, 1929.
SHEAR, M. J. AND B. KRAMER. J. Biol. Chem. **86:** 677, 1930.
SHELLING, D. H. AND M. J. GOODMAN. J. A. M. A. **102:** 669, 1934.
SHELLING, D. H. AND H. L. MASLOW. J. Biol. Chem. **78:** 661, 1928.
SHERMAN, H. C. J. Biol. Chem. **44:** 21, 1920.
J. Biol. Chem. **41:** 173, 1920.
SHERMAN, H. C. AND L. E. BOOHER. J. Biol. Chem. **93:** 93, 1931.
SHERMAN, H. C. AND H. L. CAMPBELL. J. Nutr. **2:** 415, 1930.
SHERMAN, H. C., L. H. GILLETT AND H. M. POPE. J. Biol. Chem. **34:** 373, 1918.
SHERMAN, H. C. AND E. HAWLEY. J. Biol. Chem. **53:** 375, 1922. See also J. Nutr. **2:** 415, 1930.
SHERMAN, H. C. AND F. L. MACLEOD. J. Biol. Chem. **64:** 429, 1925.
SHERMAN, H. C. AND A. M. PAPPENHEIMER. Proc. Soc. Exp. Biol. Med. **18:** 193, 1920.

SHERMAN, H. C. AND E. J. QUINN. J. Biol. Chem. **67**: 667, 1926.
SHERMAN, H. C., L. WHEELER AND A. B. YATES. J. Biol. Chem. **34**: 383, 1928.
SHIPLEY, P. G., B. KRAMER AND J. HOWLAND. Biochem. J. **20**: 379, 1926.
SHIPLEY, P. G., E. A. PARKS, E. V. MCCOLLUM AND N. SIMMONDS. Am. J. Dis. Child. **23**: 91, 1922.
SHOHL, A. T. AND H. B. BENNETT. J. Biol. Chem. **74**: 247, 1927.
SHOHL, A. T. AND H. B. BROWN. J. Biol. Chem. **84**: 501, 1929.
SHOHL, A. T., H. B. BROWN, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN. J. Biol. Chem. **98**: 215, 1932.
SHOHL, A. T. AND H. B. BENNETT. J. Biol. Chem. **76**: 633, 1928.
SHOHL, A. T., H. B. BROWN, E. E. CHAPMAN, C. S. ROSE AND E. M. SAURWEIN. J. Nutr. **6**: 271, 1933.
SHUKERS, C. F., I. G. MACY, B. NIMS, E. DONELSON AND H. A. HUNSCHER. J. Nutr. **5**: 127, 1932.
SHUKERS, C. F., I. G. MACY, E. DONELSON, B. NIMS AND H. A. HUNSCHER. J. Nutr. **4**: 399, 1931.
J. Am. Diet. Assn. **7**: 235, 1931.
SINCLAIR, R. G. J. Biol. Chem. **82**: 117, 1929.
J. Biol. Chem. **86**: 579, 1930.
SINCLAIR, R. G. J. Biol. Chem. **95**: 392, 1932.
J. Biol. Chem. **82**: 117, 1929.
SJOLLEMA, B. J. Biol. Chem. **57**: 255, 271, 1923.
Biochem. Ztschr. **200**: 300, 1928.
SJOLLEMA, B. AND L. SEEKLES. Biochem. Ztschr. **229**: 358, 1930; **264**: 316, 1933.
SMITH, H. W. J. Biol. Chem. **81**: 407, 1921.
J. Biol. Chem. **82**: 651, 1921.
SMITH, R. G. J. Pharm. Exp. Therap. **48**: 288, 1933.
SMITH, R. G. AND H. R. STERNBERGER. J. Biol. Chem. **96**: 245, 1932.
SNELL, A. M. AND C. H. GREENE. Am. J. Physiol. **92**: 630, 1930.
SOKOLOVITCH, M. Brit. J. Exp. Path. **12**: 150, 1931.
SPRETER, T. VON. Ztschr. ges. exp. Med. **85**: 19, 1932.
STANDER, H. J. J. A. M. A. **92**: 631, 1929.
STARE, F. J. AND C. A. ELVEHJEM. J. Biol. Chem. **97**: 511, 1932.
STARY, Z. AND R. WINTERNITZ. Ztschr. physiol. Chem. **182**: 107, 1929.
Ztschr. physiol. Chem. **212**: 215, 1932.
STEARNS, G. AND E. WARWEG. J. Biol. Chem. **102**: 749, 1933.
STEENBOCK, H. AND E. B. HART. J. Biol. Chem. **14**: 59, 1913.
STEENBOCK, H., E. B. HART, C. A. ELVEHJEM AND S. W. F. KLETZIEN. J. Biol. Chem. **66**: 425, 1925.
STEENBOCK, H., E. B. HART, B. M. RÜSING, C. A. HOPPERT, S. BASHEROV AND G. C. HUMPHREY. J. Biol. Chem. **87**: 103, 1930.
STEENBOCK, H., E. B. HART, B. M. RÜSING, S. W. F. KLETZIEN AND H. T. SCOTT. J. Biol. Chem. **87**: 127, 1930.
STEENBOCK, H., E. B. HART, M. T. SELL AND J. H. JONES. J. Biol. Chem. **56**: 375, 1923.
STEENBOCK, H., S. W. F. KLETZIEN AND J. G. HALPIN. J. Biol. Chem. **97**: 249, 1932.
STEPHENS, D. J. AND D. P. BARR. Proc. Soc. Exp. Biol. Med. **30**: 920, 1933.

STERKIN, E. Biochem. Ztschr. **174**: 1, 1926.
STEWART, C. P. AND J. B. S. HALDANE. Biochem. J. **18**: 855, 1924.
STEWART, C. P. AND G. H. PERCIVAL. Biochem. J. **21**: 301, 1927.
Biochem. J. **22**: 548, 559, 1928.
STRANSKY, E. Arch. exp. Path. Pharm. **78**: 122, 1915.
SWANSON, W. W. Am. J. Dis. Child. **43**: 10, 1932.
TAYLOR, N. B., H. D. BRANION AND H. D. KAY. J. Physiol. **69**: 35, 1930.
TAYLOR, N. B. AND A. FINE. Am. J. Physiol. **93**: 544, 1930.
TAYLOR, N. B. AND C. B. WELD. Am. J. Physiol. **97**: 566, 1931.
Brit. J. Exp. Path. **13**: 109, 1932.
TAYLOR, N. W. AND C. SHEARD. J. Biol. Chem. **81**: 479, 1929.
TAYLOR, W. F. AND J. E. WINTER. J. Pharm. Exp. Therap. **35**: 435, 1929.
TEICHLER, G. Pflüger's Arch. **227**: 558, 1931.
TELFER, S. V. Glasgow Med. J. **113**: 246; **114**: 10, 1930.
TEMPLIN, V. M. AND H. STEENBOCK. J. Biol. Chem. **100**: 209, 1933.
J. Biol. Chem. **100**: 217, 1933.
THEILER, A. AND H. H. GREEN. Nutr. Abst. and Rev. **1**: 359, 1932.
THEIS, R. C. AND S. R. BENEDICT. J. Cancer Res. **8**: 499, 1924.
THOMAS, R. G. Australian J. Exp. Biol. Med. **11**: 109, 1933.
THOMSON, D. L. AND L. I. PUGSLEY. Am. J. Physiol. **102**: 350, 1932.
TIGERSTEDT, C. Skandinav. Arch. Physiol. **56**: 265, 1929.
TISDALL, F. F. J. Biol. Chem. **54**: 35, 1922.
Am. J. Dis. Child. **24**: 382, 1922.
TOVERUD, G. J. Biol. Chem. **58**: 583, 1923.
TOVERUD, K. U. AND G. TOVERUD. Norsk. Mag. Laegev. **92**: 677, 1931.
Norsk. Mag. Laegev. **94**: 18, 1933.
TREDELENBURG, P. AND VON W. GOEBEL. Arch. exp. Path. Pharm. **89**: 171, 1921.
TUNG, P. C. Chinese J. Physiol. **6**: 277, 1932.
UNDERHILL, F. P. AND O. E. CLOSSON. Am. J. Physiol. **15**: 321, 1905.
UNDERHILL, F. P. AND A. DIMICK. J. Biol. Chem. **58**: 133, 1923.
UNDERHILL, F. P. AND E. G. GROSS. J. Biol. Chem. **81**: 163, 1929.
UNDERHILL, F. P., E. G. GROSS AND W. COHEN. J. Metab. Res. **3**: 679, 1923.
UNDERHILL, F. P. AND T. C. JALESKI. J. Biol. Chem. **101**: 11, 1933.
UPDEGRAFF, H., D. M. GREENBERG AND G. W. CLARK. J. Biol. Chem. **71**: 87, 1926.
VAN SLYKE, D. D. AND J. SENDROY. J. Biol. Chem. **84**: 217, 1929.
VAUGHAN, K. Brit. Med. J. **1**: 413, 1926.
WACKER, L. Biochem. Ztschr. **196**: 426, 1928.
Klin. Wchnschr. **8**: 244, 1929.
WALKER, A. M. J. Biol. Chem. **101**: 269, 1933.
WALKER, B. S. J. Lab. Clin. Med. **17**: 347, 1932.
WALKER, B. S. AND E. W. WALKER. J. Lab. Clin. Med. **18**: 164, 1932.
WALSH, E. L. AND A. C. IVY. Proc. Soc. Exp. Biol. Med. **25**: 839, 1927.
WARBURG, E. J. Biochem. Ztschr. **178**: 208, 1926.
WATCHORN, E. Quart. J. Med. **18**: 288, 1924.
Brit. J. Exp. Path. **7**: 120, 1926.
Biochem. J. **24**: 1560, 1930.
WATCHORN, E. AND R. A. MCCANCE. Biochem. J. **26**: 54, 1932.
Biochem. J. **27**: 1107, 1933.

WATSON, R. H. Australian J. Exp. Biol. Med. Sci. **11**: 197, 1933.
WEAVER, W. K. AND C. I. REED. J. Biol. Chem. **85**: 281, 1929.
WEECH, A. A., A. GOETTSCH AND E. B. REEVE. J. Clin. Invest. **12**: 1021, 1931.
WEINSTEIN, A. A. AND S. WEISS. Arch. Int. Med. **48**: 478, 1931.
WELLS, H. S. Am. J. Physiol. **101**: 421, 435, 1932.
WESSELOW, O. L. V. DE. Quart. J. Med. **16**: 341, 1923.
Lancet **2**: 163, 1923.
WHEELER, W. P. Exp. Station Record **43**: 574, 1920.
WHEELON, H. Endocrinol. **13**: 363, 1929.
WHITE, J. C., M. E. FIELD AND C. K. DRINKER. Am. J. Physiol. **103**: 34, 1933.
WIDDOWS, S. T., M. F. LOWENFELD, M. BOND AND E. I. TAYLOR. Biochem. J. **24**: 327, 1930.
WILDER, T. S. J. Biol. Chem. **81**: 65, 1929.
WILKINS, W. E. AND G. E. CULLEN. J. Clin. Invest. **12**: 1063, 1933.
WINTER, J. E. AND C. H. RICHEY. J. Pharm. Exp. Therap. **41**: 245, 1931.
J. Pharm. Exp. Therap. **42**: 179, 1931.
WINTER, J. E., C. H. RICHEY AND H. G. BARBOUR. J. Pharm. Exp. Therap. **38**: 343, 1930.
WOKES, F. J. Pharm. Exp. Therap. **43**: 531, 1931.
WOOLEY, J. B. AND H. ROSS. U. S. Pub. Health Repts. **47**: 380, 1932.
WRIGHT, H. N. AND D. W. COWAN. Proc. Soc. Exp. Biol. Med. **27**: 950, 1930.
YAMAWAKI, S. Arch. exp. Path. **136**: 1, 1928.
ZUCKER, T. F. Proc. Soc. Exp. Biol. Med. **18**: 272, 1921.

ADDENDA. Since the completion of this manuscript a number of publications have appeared which contain material of great importance to certain of the topics treated in this review, making it desirable to give a brief account of their contents.

By quantitatively standardizing the response of the isolated frog heart to calcium ion, McLean and Hastings have developed an important tool for the investigation of many physiological problems which concern calcium. With the frog heart method, these authors have been able to measure directly the calcium ion concentration of blood plasma, cerebrospinal fluid, and other biological fluids obtained from human subjects and experimental animals. The results of the measurements on human blood plasma are in good agreement with the values for the diffusible calcium content obtained by membrane distribution methods. Support is thereby gained for the contention that the calcium which is not combined with the colloids of the plasma is present almost totally as calcium ions. Their measurements also show that the calcium ion concentration of the blood remains unchanged in rickets, is lowered in the hypocalcemia following thyroparathyroidectomy, and is increased during the hypercalcemia produced by injection of parathyroid extract.

McLean and Hastings have also probed the problem of establishing a

mathematical relationship between the calcium and protein content of the blood plasma. They reached the conclusion that the ionization of calcium is determined chiefly by the protein content of the plasma, and that as a first approximation, the relation between calcium and protein can be described by a simple mass law equation. The ionization constant of the calcium proteinate of human blood, based on this relationship, was estimated to have the value of $10^{-2.22 \pm 0.07}$ at 25° and pH 7.35. This constant, however, is open to question since it depends upon the validity of the assumption that the calcium binding capacity of the serum proteins is equivalent to their total base titration value. This assumption is not wholly probable. A means of evaluating the calcium binding equivalence of the proteins has been suggested by Greenberg, Larson and Tufts. From this work it becomes apparent that the chief value of an equation which relates calcium to the protein content and other variables lies in the possibility of being able to estimate the calcium ion concentration. McLean and Hastings state that this may be calculated in human body fluids by means of their equation with a degree of accuracy which is probably as great as that which direct observation by the frog heart method permits.

McLean, Hastings and co-workers also have measured the ionization of the citrates of the alkaline earth elements and have obtained important evidence favoring the existence of negatively charged metal-containing citrate ions. The mode of combination of the citrate with the metal to give this type of ion probably follows the equation:

$$Ca^{++} + Cit^{\equiv} \rightarrow Ca\ Cit^{-}.$$

Further important work has also appeared on the effects which are produced by magnesium deprivation. In measurements which were carried out on the mineral metabolism of dogs restricted to a very low magnesium diet, Kruse, Schmidt and McCollum found, as would be expected, that the magnesium excretion was decidedly diminished throughout the entire survival period of the animals. The metabolism of calcium in this condition was very interesting. This element was retained markedly during the first five week period of the deficiency and then was eliminated in progressively increasing amounts during the latter part of the experiment. Both calcium and magnesium were excreted to a greater extent through the intestines than in the urine. It is suggested that the calcium retention in the early stages of the deficiency is due to an antagonism between calcium and magnesium, while the later reversal of this process is probably the result of nutritional

failure. A progressive increase in the excretion of urinary nitrogen is offered in support of this theory.

The chemical changes produced in the bones of rats maintained on the extremely low magnesium diet were measured by Orent, Kruse and McCollum. The authors observed that the bones of these animals were unusually heavy and overly abundant in their absolute content of ash, calcium and phosphorus. The bone was particularly noteworthy with respect to its abnormally high percentage of calcium. The increment appears to be limited mainly to the first five days on the diet. The changes in the bone are also attributed to the physiological sway of magnesium over calcium.

Although the animals received an insignificantly small amount of magnesium, their bones showed an actual accumulation of this element. The absolute content of magnesium was reduced to about half of the level found in normal bone. Orent, Kruse and McCollum suggest that the extra magnesium in the bones comes from the soft tissues of the body. However, analyses which are being carried out by Greenberg and Tufts show that such tissues as muscle, brain, liver, and kidney of magnesium deficient rats show no noteworthy reduction in their magnesium content.

The failure to raise calves to maturity on a ration of whole milk or milk supplemented with cod liver oil and iron, copper and manganese is attributed by Duncan, Huffman and Robinson to a failure of magnesium metabolism which results in a magnesium deficiency. The failure to utilize magnesium, even though its intake was between 5.4 and 13.7 mgm. per kilo of body weight, is believed by the above authors to result from the lack of roughage in the milk diet. These calves showed a consistently low plasma magnesium level, although not so low as was found in the magnesium deficient animals of Kruse, Orent and McCollum. The calves lost their appetite, became temporarily blind, and went into convulsive attacks which often terminated fatally.

REFERENCES

McLean, F. C. and A. B. Hastings. J. Biol. Chem. **107:** 337, 1934; **108:** 285, 1935.

Hastings, A. B., F. C. McLean, L. Eichelberger, J. L. Hall and E. Da Costa. J. Biol. Chem. **107:** 351, 1934.

Greenberg, D. M., C. E. Larson and E. V. Tufts. Proc. Soc. Exp. Biol. Med. **32:** 647, 1935.

Kruse, H. D., M. M. Schmidt and E. V. McCollum. J. Biol. Chem. **106:** 553, 1934.

Orent, E. R., H. D. Kruse and E. V. McCollum. J. Biol. Chem. **106:** 573, 1934.

Duncan, C. W., C. F. Huffman and C. S. Robinson. J. Biol. Chem. **108:** 35, 1935.

THEORIES OF BLOOD COAGULATION

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Clotting is the most striking and best known property of blood. It is, indeed, a remarkable phenomenon. One can realize that nature had a difficult problem to solve in creating a circulating medium for the animal body that would remain entirely liquid while in the blood-vessels, but would promptly set into a gel as soon as the vessels were wounded, and the blood was in danger of being lost. The stages through which this property, as exhibited in the higher animals, was developed during the course of evolution are not known, in fact have not been extensively studied, although it would seem probable that something of its history would be revealed by a comparison of the conditions existing in the different animal phyla. It goes without saying that in man and the higher vertebrates it has been at all times a subject for investigation and speculation, and when physiology became finally established as an experimental science, in the early part of the 19th century, it offered itself naturally as a suitable subject for the application of the experimental methods of physics and chemistry. Very many workers have attempted to unravel the chemical and physical mechanisms responsible for the sudden change of condition from a sol to a gel, but the process has proved to be an intricate one involving a number of different factors. While much progress has been made in determining the nature of these factors and the way in which they interact to produce the end result, the complete story is still unknown. There has been and still is a wide divergence of opinion among special workers in the subject in regard to the interpretation of known facts. A large mass of experimental data has been collected, but no one of the numerous theories proposed to correlate and explain the observed facts has been so demonstrably adequate as to meet with general acceptance.

The theories actually under consideration today cross and overlap to an extent that makes it difficult for those who are not special students of the subject to disentangle one from the other or to determine which has the greater degree of probability. From time to time general reviews have been published which attempt to summarize the important

data and to compare critically the various theories proposed. Among the most recent of these reviews attention may be called especially to those prepared by Wöhlisch (1929), Fuchs (1930, 1933) and Zunz (1934). For the purposes of the specialist in the subject the contribution by Wöhlisch is particularly complete. Outside the general sources consulted the author brings under review some nine hundred special papers. All this great mass of material is classified, compared and critically discussed with great ability and fairness. It would serve no useful purpose to repeat work so well done. The present brief review, therefore, attempts only to call attention to the more important work that has appeared since Wöhlisch's paper and to present the status quo of the subject, from the point of view of the writer, for the information of workers in other fields rather than for the use of the specialist.

What may be called the orthodox or classical theory of coagulation began with the work of Alexander Schmidt (1861). His initial experiments were based upon the preceding work of Buchanan (1845) and Denis (1859), as has been pointed out by Gamgee (1880) in his excellent review of the work of that period. Buchanan demonstrated that a substance is present in serum, or may be extracted from blood-clots, which when added to the incoagulable exudates of the body, hydrocele and pericardial liquids, causes them to clot. Denis proved that an albuminous substance, designated by him as plasmine, may be precipitated from blood-plasma by saturation with sodium chloride which when redissolved undergoes spontaneous coagulation, thus indicating that the plasma contains a soluble precursor of the fibrin of the clot. Schmidt (1872) was able to show that the substance present in serum, or extractable from a clot, is a definite body that acts, he believed, after the manner of a ferment upon some of the albuminous material of the plasma, and, with the coöperation of the inorganic salts, converts it to fibrin. He designated this substance first as fibrin ferment, later as thrombin, and showed that it is formed, after the shedding of the blood, from an inactive precursor, prothrombin, which is present in the plasma of the circulating blood. In his original theory Schmidt assumed that two of the proteins of the blood, fibrinogen and fibrinoplastic substance (serum globulin) are concerned in the production of fibrin, but somewhat later (1880, 1883) Hammarsten simplified his theory by demonstrating that the so-called fibrinoplastic substance takes no essential part in the process, and that the fibrin of the clot is formed by a reaction between fibrinogen and thrombin, the latter being present normally only in shed blood. This view has been accepted by the great majority of succed-

ing workers in the subject. It constitutes the common basis or cornerstone upon which most of the theories of coagulation have been erected, and may be designated in general as the thrombin theory of coagulation. Those who have accepted it have differed among themselves chiefly in regard to the mode of production of thrombin from its inactive precursor in the circulating blood.

There can be no question whatever that when thrombin is added to a solution of fibrinogen fibrin is formed and deposited as a typical clot, but some few workers have contended that this reaction does not actually take place in the normal clotting of blood, or at least, it is not the essential change. The first to express such an antagonistic view was Wooldridge (1883–1887). As the result of his extensive investigations he came to the conclusion that clotting is due to a reaction between two protein lecithin compounds, both present in circulating blood, which he designated as A-fibrinogen and B-fibrinogen. The latter is found only in the blood and is spoken of specifically as blood fibrinogen. The former, however, occurs also in the tissues and is designated, therefore, in general as tissue fibrinogen. Thrombin is formed as one of the products of the reaction, but it does not play a primary rôle in the initiation of the act of clotting. The nature of the reaction between the two fibrinogens was not specified other than to say that the contained lecithin is probably an active factor in the change that takes place.

Wooldridge's experimental data were new and important, but his interpretation or his theory of clotting seemed to be inadequate in the light of other known facts and its failure to account satisfactorily for the fluidity of the circulating blood, and the sudden change in this condition that occurs when the blood is shed. It met with practically no confirmation from other workers, until it was revived in this country by Mills (1921). This author recognizes the existence of thrombin in shed blood and admits that it reacts with fibrinogen to form thrombin, but in agreement with Wooldridge he considers that normal clotting in wounds is due primarily to a reaction between a tissue fibrinogen, which is defined as a protein cephalin compound, and the fibrinogen of the blood-plasma. It seems unfortunate that Mills adopted Wooldridge's term tissue fibrinogen to designate the factor furnished by the tissue cells. As used by the latter it was employed apparently in its etymological sense to indicate that the substance was a factor in the generation of fibrin, but since that time the name fibrinogen has become accepted as the designation of a specific blood protein which, so far as is known, does not occur in the tissue cells. It is not claimed or, at least,

it is not proved that so-called tissue fibrinogen contains this particular protein. Mills defines it as consisting of a protein nucleus with a number of attached phospholipin (cephalin) groups, each of which, through the agency of a calcium ion, is capable of binding a molecule of blood fibrinogen, giving, therefore, the possibility of the formation of fibrin with a varying molecular weight.

It has long been known that tissue extracts, aqueous or alcoholic, when added to blood greatly hasten its coagulation. This effect is especially noticeable when dealing with blood plasmas that are slowly coagulable or that have been deprived of the power of spontaneous coagulation by the action of an anticoagulant. The result is explained usually by the presence in the tissues of a so-called thromboplastic substance which causes the activation of the prothrombin of the plasma. If one wishes to show that the tissues contain a protein compound that, in the presence of calcium, can actually unite with the fibrinogen of the blood to form fibrin, it is not sufficient simply to add the tissue extract and calcium to a plasma, since the latter contains prothrombin. The crucial experiment is to show that tissue extract and calcium cause fibrin formation when added to a solution of pure fibrinogen, without the possibility of the intervention of prothrombin or thrombin. Mills believes that he has obtained this reaction, but other observers report negative results. Wooldridge himself was unable to cause clotting of fibrinogen by tissue extract and explained his failure by the assumption that fibrinogen obtained by salting out plasma is denatured or altered as compared with the fibrinogen of the blood. Fuld and Spiro (1904), and Morawitz (1904) state that addition of tissue extract to purified fibrinogen in the presence of calcium gives no clot. More recently the question has been studied with especial care by Smith, Warner and Brinkhous (1934). Like Mills, they made use of lung extract on account of its high degree of thromboplastic potency. They found that if the blood is not completely removed from the lungs by perfusion the extract will contain remnants of the blood prothrombin. When proper care was taken to avoid this contamination and the fibrinogen solution likewise was freed from prothrombin by the use of special methods, then a mixture of lung extract, fibrinogen and calcium failed to clot. Addition of prothrombin caused prompt clotting. The evidence at present, therefore, tends to disprove the Wooldridge-Mills conception of the origin of fibrin.

Stuber and Lang (1930) have also proposed a theory of clotting in which thrombin takes no part. They differ from practically all other

workers in the subject by denying that calcium is concerned in the process. Their thesis is that blood glycolysis is the ultimate cause of clotting. The lactic acid produced by the glycolysis is the immediate agent, the *causa movens* of the formation of fibrin, acting by virtue of the increased concentration of hydrogen ions which causes the precipitation or flocculation at its isoelectric point of the fibrinogen of the blood plasma. The evidence for this theory in general terms is based upon the complete parallelism which the authors assert exists between the rate of glycolysis in shed blood and the coagulation time. By quantitative examination of the sugar of the blood, after its escape from the vessels, they find that it disappears in relation to the time of coagulation, and conditions that shorten or lengthen the coagulation time have an identical effect upon the velocity of glycolysis. This relationship is exhibited particularly in the action of the known anticoagulants, which they find tend to inhibit the first or anaerobic phase and completely block the oxidation phase. In this latter effect they find an explanation of the action of anticoagulants (Stuber, 1930), but it is difficult to see the force of the argument since suspension of the oxidation phase should permit accumulation of acid. They find an explanation of the slow clotting of hemophilic blood in the fact that it contains more fluorine than normal blood and fluorides are known to depress the process of glycolysis. A similar excess of fluorine in the blood of the goose is assumed to explain its delayed clotting when protected from contact with wounded tissue, but they offer no adequate explanation for the prompt and firm coagulation that ensues when the blood flows over wounded tissue or is mixed with tissue extract, other than the general statement that tissue juice or extract alters the surface tension relations. The action of oxalates in preventing clotting is not in their opinion the result of the precipitation of the calcium, but is caused by the effect of the excess of oxalate added. The oxalate forms a salt complex of some kind with fibrinogen that brings about such a degree of ionization and dispersion of the fibrinogen as to make its precipitation impossible. They emphasize the fact that to prevent clotting much more oxalate must be added than is necessary to precipitate the calcium, and they give evidence to show that when oxalate is added in amounts more than sufficient to precipitate the calcium, but not enough to prevent clotting, analysis of the clot and contained serum, after removal of the calcium oxalate, shows that no calcium is present. That is to say, clotting has taken place in the absence of calcium (Stuber and Focke, 1924).

This general result is in accord with the experience of other workers

Vines (1921) found that it takes about three times the amount of oxalate theoretically sufficient to precipitate the calcium to keep the blood from coagulating. On the other hand when the oxalated plasma is recalcified coagulation occurs before all of the excess of oxalate is precipitated. Similar findings have been reported by Loucks and Scott (1929) and by Scott and Chamberlain (1934). According to the latter clotting may begin on recalcification when only enough calcium has been added to combine with $\frac{1}{20}$ of the oxalate present, and solid clotting may occur when only $\frac{1}{3}$ of the oxalate has been neutralized. There is apparently no fixed relationship between the amount of calcium and the beginning and extent of the clotting. There would seem to be no question that oxalates have an effect upon the clotting system of the plasma, the prothrombin fibrinogen relationship, other than the simple removal of the calcium, which is not at present understood. It may be noted, however, that the nearly universal belief that calcium plays an essential rôle in clotting is not based solely upon the oxalate effect. Wöhlisch and Paschkis (1924), making use of a magnesium sulphate plasma, were able to dialyze off the calcium and after removal of the magnesium sulphate, also by dialysis, obtained a plasma that did not clot spontaneously but did so when calcium was added. So also preparations of prothrombin obtained by any of the accepted methods are wholly unable to induce clotting in fibrinogen solutions or in calcium free plasmas, but become active almost immediately when calcium is added. It is well known that under experimental conditions clotting may take place without calcium, since prothrombin may be activated to thrombin by other reagents, such as chloroform, or may even under certain conditions pass over to thrombin spontaneously, but there can be little or no doubt that in the normal clotting of blood calcium ions play an active and essential rôle.

If one grants that the quantitative work of Stuber and Lang upon glycolysis in the blood in the period preceding coagulation is accurate, the further assumption that the clotting is due directly to the increased hydrogen ion concentration is theoretical and not wholly in accord with other facts. As Wöhlisch (1929) has pointed out acid precipitation of fibrinogen is a reversible process, whereas normal clotting is an irreversible reaction. It might be added that acid precipitation does not give the gel structure that is characteristic of normal clotting and of thrombin precipitation of fibrinogen. However, the analytical results upon the velocity of glycolysis have failed of confirmation in the hands of other workers. Hartmann and Kühnau (1930) contest the fundamental

statement upon which the theory rests. In the interval preceding clotting, even when this period is prolonged for as much as 40 minutes by the use of paraffined vessels, there is no measurable diminution in the concentration of the blood sugar. On the other hand they find that in hemophilic blood and in blood kept from clotting by various anticoagulants a gradual glycolysis takes place without coagulation. By the use of a special method of electro-ultrafiltration they succeeded in removing sugar from oxalated plasma. This sugar free plasma clotted readily upon the addition of calcium chloride. Partos (1932) criticizes the analytical methods used by Stuber and Lang, but making use of the same methods he was unable to determine any definite direction of change in the sugar concentration of blood examined at intervals of 7 and 12 minutes after shedding. In some cases there was a decrease, but in others not, or an increase. He makes the categorical statement that there is no definite relation between the process of glycolysis and coagulation, although he finds that in hyperglycemic blood coagulation time is shortened and in hypoglycemia it is retarded. Fuchs and von Falkenhausen (1931) report similar negative results. While the glycolytic theory offers a definite and relatively simple explanation of clotting it ignores or contradicts some well attested facts, and for this reason its value will remain uncertain until further tested at the hands of other workers.

Among the older theories that of Nolf (1906) may be classified also as belonging to the non-thrombin group. His view, in brief, is that there exist in the plasma three colloidal substances whose union results in the formation of fibrin. Two of them, fibrinogen and thrombogen, are protein bodies which are formed in the liver, while the third, thrombozyme, a proteolytic enzyme, is derived from the leucocytes and platelets and the endothelial cells of the blood and lymph vessels. He recognizes the existence of thrombin, but considers it as a compound of thrombogen and thrombozyme with fibrinogen, a product, therefore, similar to fibrin. He speaks of it as a soluble form of fibrin and regards it as a result of the process of coagulation rather than as the cause. In its complete form his theory is intricate and speculative, particularly in regard to what he considers the normal and functional deposition of fibrin upon the surface of the living cells and its subsequent use in their nutrition. His interesting speculations have not been confirmed by subsequent workers.

It would appear from this brief resumé that the efforts to show that thrombin is not an essential factor in coagulation have not been entirely

successful. The balance of evidence tends strongly to support the belief that the fundamental process in clotting is the reaction between thrombin and fibrinogen. In all probability it is in the development of the thrombin theories that the solution of the problem is to be expected. Some of the controversial points that have arisen in connection with these theories may be brought out by a consideration of the several factors that are believed to be concerned in the process of clotting.

Fibrinogen. All theories agree that the fibrin is derived from fibrinogen. Its properties as a protein belonging to the globulin group are well known. It is the least soluble of the proteins of the blood and the one that most easily becomes denatured. Its isoelectric point has been variously placed by different observers, but the careful work of Mainzer (1932) would seem to show that when isolated from the plasma by the method of Hammarsten its indifferent point in buffered solutions as determined by cataphoresis, lies at a pH of 5.15, while as determined by maximum of instability (flocking) it is at pH 4.36. Its concentration in the blood lies around 400 mgm. or less per 100 cc. A recent determination gives from 250 to 350 mgm. as the normal limits in dog's blood (Jones and Smith, 1930). Many observers agree that the fibrinogen is formed in the liver. When this organ is removed the concentration decreases gradually so that within 12 to 20 hours some 20 to 50 per cent disappears. It is to be presumed that under normal conditions the protein is constantly formed and is constantly utilized in some functional activity of the body, but nothing is known definitely as to the purposes it subserves or the organ or organs responsible for its consumption. Some isolated observations indicate that the lungs may be concerned in its metabolism (Pavlov, 1887; Bohr, 1888; Doyon, Morel and Kareff, 1905). Under normal conditions all of the fibrinogen is used up in clotting, none is found in the serum. Under experimental conditions the clotting may be partial or take place in successive stages.

The relation of the thrombin to fibrinogen is highly specific. There is no good evidence that it reacts in a similar way with any other protein. Del Baere (1932) has claimed that while thrombin does not cause precipitation of the other serum proteins it does react with them in some way, as is indicated by a change in their osmotic pressure, but his evidence for this assertion is very inconclusive. The nature of the reaction between fibrinogen and thrombin is not definitely known. Many hypotheses have been suggested but none of them has been supported by convincing experiments. Hammarsten in accordance with the usual belief considered that thrombin acts as a hydrolytic enzyme and causes

cleavage of the fibrinogen molecule with the production of the insoluble fibrin and of a soluble protein which can be detected in the serum, but this view was not well supported by his own later experiments nor by those of other workers. This view is still maintained by Mellanby (1933) who speaks of thrombin as a proteoclastic enzyme that splits fibrinogen with the formation of fibrin and a soluble serum globulin.

Waldschmidt-Leitz and his co-workers (1929) explain thrombin as a proteolytic enzyme related to pancreatic trypsin. As the latter is activated by enterokinase so the former is activated by thrombokinase. This view is founded on the fact that hirudin is destroyed by trypsin-kinase, and that trypsin has some facilitating effect on clotting. Assuming that hirudin is a proteolytic substrate and that its action in preventing clotting is due to its combination with thrombin, they infer that the latter must be a proteolytic enzyme. One can not feel that the logic of the argument is convincing. A somewhat similar argument from analogy is offered by Kühnau and Morgenstern (1934). They find that glutathione by a reaction with thrombin inhibits clotting at reactions less than pH 7.4, and has the contrary effect at pH 7.4. The former effect is strengthened by traces of copper and the latter by iron. Since copper and iron have similar effects upon the action of sulphydril compounds upon known hydrolytic enzymes such as kathepsin and papain they assume that thrombin belongs to the same group. Making use of an optical method Klinke (1931) also comes to the conclusion that thrombin is an enzyme. By observation upon the Tyndall effect, as determined by a nephelometer, he measured the change in dispersion of the fibrinogen during its conversion to fibrin under the influence of thrombin. The curve constructed from these data corresponds with that characteristic of ferment reactions. In a preliminary communication by Fuchs and Zakrzewski (1934) the authors state that by using a colorimetric micromethod for the determination of nitrogen they have been able to demonstrate an increase in non-protein nitrogen as a result of the coagulation reaction. Analysis of their results, in fact, indicates two phases, first a reaction in which the non-protein nitrogen is decreased and second, one in which it is increased. The latter factor being the larger the final result is an increase in non-protein nitrogen. They connect the first reaction with the activation of prothrombin and the second with the action of thrombin on fibrinogen, and consider that the result is further proof of the hydrolytic action of thrombin. While the view that the conversion of fibrinogen to fibrin is a hydrolytic process is held probably by a majority of workers in the subject it is evident from the

brief references given, that convincing experimental proof is still lacking. The products of the hydrolysis can not be separated and determined, nor does fibrinogen respond to other known hydrolytic agents or enzymes with the formation of a fibrin gel.

Other specific or general theories have been advanced particularly by recent workers who have approached the problem from the standpoint of colloidal chemistry and interpreted the changes of coagulation in terms of variations in degree of dispersion of the fibrinogen sol. Hekma, for example (1923), in one form of his theory considered the fibrinogen as an alkalihydrosol of fibrin dispersed as amicrons. Thrombin, liberated from the platelets and leucocytes, acts as an agglutinin to these amicrons causing them to adhere and form needles or crystals, the process being accompanied or preceded by an independent act of dehydration. In a later form of his theory (1931) he adopts the hypothesis of Mellanby and Pickering that the fibrinogen, or part of it at least, exists in the circulating blood in a complex containing, beside the fibrinogen, globulin, albumin and thrombin or prothrombin. The kinase liberated from tissue cells or blood-platelets causes this complex to dissociate. The free fibrinogen thus formed deposits as fibers or needles which form a gel. The prothrombin is changed to thrombin and then acts as a precipitin towards the fibrinogen in solution, aggregating it out as needles or fibers which form a gel. This process is reversible whereas normal clotting is not. He assumes, therefore, the production of an agglutinin, probably a calcium compound, from the erythrocytes, which acts as a cement to bind together the fibrin fibers and needles and form the irreversible fibrin of the normal clot. Hekma's views seem to be entirely speculative and are not supported by direct experimental work. They constitute simply a possible interpretation from a physico-chemical point of view of the phenomena of coagulation.

A more significant contribution is reported by Del Baere (1932). By means of cataphoresis experiments with a special arrangement of his coagulating solutions he believes that he demonstrates that thrombin travels toward the negative pole and carries, therefore, a positive charge. Assuming that the fibrinogen has a negative charge he explains the deposition of fibrin as a flocking out of the fibrinogen in consequence of its electrical adsorption of thrombin. He recognizes that a similar reaction should occur between the thrombin and the negatively charged globulin or albumin, and gives some inconclusive evidence of a combination of this kind resulting in a change in the osmotic pressure of the globulin or albumin. Del Baere's results seem to be open to other

interpretations and can scarcely be accepted without further confirmation, especially as other workers who have used the method of cataphoresis with solutions of thrombin have found that its charge varies with the reaction of the solution without influencing its coagulating action (Howell, 1916).

Stuber and Lang in their theory of coagulation, as previously stated, assume that fibrinogen in clotting is flocked out at its isoelectric point by the lactic acid resulting from the glycolysis of the blood sugar; while Fischer (1933) in his recent new theory of coagulation adopts the view that thrombin as a calcium lipoid protein unites chemically with fibrinogen to form fibrin.

It would seem that this important problem of the nature of the reaction between thrombin and fibrinogen is still unsolved. No definite advance in our knowledge has been made since the original studies of Hammarsten toward the end of the last century.

Prothrombin. The usual view in regard to prothrombin is that it is a protein, present in the plasma of the circulating blood, which is readily converted to thrombin by the action of calcium and tissue extract. Its ready conversion to thrombin implies that in the circulating blood it is protected in some way to ensure the fluidity of the blood. Various hypotheses have been suggested to explain its stability under the normal conditions of the circulation. According to Morawitz its inactivity is due to the lack of activating substance, the thrombokinase of tissue extract. Bordet designates the prothrombin found in serum as serozyme and assumes that in the circulating blood it exists in a protected form which he calls proserozyme. Mere contact with a foreign surface, that is, a surface that the blood can wet, is sufficient, in the presence of calcium, to change the proserozyme to serozyme and lead to clotting. This hypothesis is made improbable by the fact that several observers have, by special means, obtained normal plasma free from corpuscular elements and found that it is entirely stable when brought into contact with a foreign surface, such as glass. No clotting ensues from the action of this physical factor alone, provided the platelets and other formed elements have been removed. Howell believes that in circulating blood prothrombin is protected by combination with an anticoagulant or inhibiting substance, heparin or antiprothrombin, which he has shown to be present in blood and Fuchs suggests that what Bordet calls proserozyme is in fact this prothrombin-antiprothrombin combination. Mellanby, Pickering and also Hekma conceive that in the normal blood prothrombin is protected because it exists in a colloidal complex with

fibrinogen, serum globulin and albumin. Exposure to a foreign surface or to the action of tissue extract causes the dissociation of this complex with the liberation of the prothrombin and its subsequent activation. Fischer in his recent work seems to dismiss prothrombin as a specific precursor of thrombin. Under the term prothrombin he designates proteins of a globulin nature found in blood and the tissues generally which by combination with a lipoid element in tissue extract become denatured in the sense that their isoelectric point is changed from the acid side to near the neutral point, this change being a necessary prerequisite for the subsequent combination with fibrinogen to form fibrin. In the non-thrombin theories of coagulation, such as the theory of Mills or of Stuber and Lang, prothrombin, of course, loses its significance entirely. There can be no doubt, however, that a precursor of thrombin, to which we can give the convenient name of prothrombin, actually exists in the circulating blood and also in the serum after the blood has clotted. Eagle (1935a) gives quantitative determinations to show that the amount of thrombin formed in a mixture of prothrombin, calcium and thromboplastic extract varies directly with the prothrombin (plasma factor) and is independent of the other two factors.

Several methods have been described for obtaining this substance from blood plasma.

In Mellanby's method, as recently modified (1930), oxalated plasma is diluted tenfold with water and then precipitated by addition of dilute acetic acid to pH 5.3. The precipitate which contains fibrinogen and serum globulin as well as prothrombin is treated with a dilute solution of calcium bicarbonate which dissolves out the prothrombin. The solution is again precipitated with dilute acetic acid and the precipitate is centrifugalized off and dried with acetone. In the preparation of serozyme Bordet and Delange (1914) made use of its property of being readily adsorbed. An oxalated plasma, freed as far as possible from formed elements by centrifuging, is treated with a suspension of tricalcium phosphate. After time is allowed for adsorption the mixture is centrifugalized and the precipitate, after washing, is suspended in salt solution and submitted to the action of a stream of carbon dioxide. This dissolves the phosphate and leaves the prothrombin in solution. Fuchs (1928) has improved the method by using magnesium hydroxide in place of the tricalcium phosphate. In Howell's method (1914) a clear oxalated plasma is precipitated in small lots of 5 cc. by an equal volume of acetone. The precipitate is filtered off rapidly by means of a suction pump, washed once with ether and then spread in a thin layer

on the filter paper and dried in a current of air from a fan. In this form the material keeps indefinitely. When extracted with water made slightly alkaline a solution is obtained which has no action on fibrinogen. If a calcium salt is added thrombin is formed at once and the solution causes rapid clotting in solutions or plasmas containing fibrinogen. The solutions prepared by these several methods give protein reactions, but these may be due of course to proteins present as impurities. There is no evidence that prothrombin has been obtained in pure form and we have therefore no decisive proof of its chemical nature. Certain differences in the reactions of the prothrombin as prepared by these various methods may be due to the effect of accompanying impurities.

Granting the existence of prothrombin and the part it takes in normal coagulation two important questions in regard to it have been under discussion for a long time, and still remain unsettled. One is in regard to the source of the prothrombin, and the other is the nature of the change that takes place when it is converted to thrombin. Schmidt in his pioneer work upon fibrin ferment evidently believed that it may be obtained from other sources than the blood, the lymph glands, for example, or the corneal tissue, and to judge from the literature (see Bonne, 1889), it was thought generally at that period that fibrin ferment or thrombin constitutes one of the products of degenerative changes in any animal cell. The liberation of an excess under pathological conditions was called upon to explain certain symptoms connected with an obstructed circulation. Later, however, in consequence of the work of Morawitz, Bordet and others the belief became established that prothrombin occurs only in the blood and lymph, or that this is its sole source in coagulation. The disintegration of the platelets that precedes clotting led naturally to the view that they give origin to prothrombin and Morawitz (1904b) was able to show that when the platelets are collected by differential centrifugalization, and washed and extracted, prothrombin is obtained. This result has been contested by Bordet and others, but most workers who have repeated the experiment have confirmed Morawitz's statement. We are justified, therefore, in considering the platelets as the probable source of the prothrombin found in the blood. In addition, in experiments involving perfusion of bone marrow Drinker and Drinker (1916) have given seemingly positive evidence of the formation of prothrombin in that tissue. Efforts to ascertain which of the cellular elements of the marrow is responsible for the production appeared to rule out the leucocytes, lymphocytes, myelocytes, nucleated red cells and the endothelial cells and to point to the megacaryocytes as the source.

In recent years Fuchs and also Fischer have revived the older view that prothrombin is furnished by all or many of the tissues. Fischer and Schmitz (1933) state that thrombin may be obtained from all protein containing tissue extracts. Kraus and Fuchs (1929) report careful experiments in which they obtained prothrombin from the muscular tissue of the rabbit. The animals were bled, the leg muscles were removed, cut into small pieces, frozen with liquid air and then powdered. When this powder was added to an oxalated plasma, from which prothrombin had been adsorbed by magnesium hydroxide, clotting occurred. In two respects this experiment seems to be open to criticism. The muscles after bleeding must still have contained some blood and it is possible that the prothrombin came from this source rather than from the muscle tissue. It will be remembered that in the work of Smith, Warner and Brinkhous, previously referred to, this difficulty was encountered. Extracts made from lung tissue of an animal that had simply been bled caused clotting of fibrinogen solutions in the presence of calcium, whereas when the lungs, after thorough bleeding, were also perfused, the extracts subsequently made although very rich in thromboplastic substance, gave no clot when added to a solution of pure fibrinogen in the presence of calcium. Evidently the clotting in the first case was caused by thrombin derived from the prothrombin of the residual blood in the lungs. The same objection applies to Fischer's experiments, since he seems to have used a cold saline extract of the minced breast muscles of fowls that had been bled only and not perfused. In the second place Kraus and Fuchs used for their fibrinogen solution an oxalated plasma from which prothrombin had been removed by adsorption with magnesium hydroxide. There is no doubt that this reagent is most effective in adsorbing prothrombin, but so far as the writer knows there is no convincing proof that the adsorption is complete.

With the exceptions previously mentioned most workers in the subject agree that the calcium ion takes part normally in the activation of prothrombin. When prepared from calcium free plasma prothrombin does not cause clotting if added to a solution of fibrinogen or to an oxalated plasma. In the presence of calcium, however, clotting takes place promptly. The nature of the reaction between calcium and prothrombin has not been definitely determined. It is still a matter of controversy. The older view advocated by Pekelharing (1892) that thrombin is a calcium compound and that in the reaction with fibrinogen the calcium is transferred to the fibrinogen or to a portion of the fibrinogen molecule giving an insoluble compound, fibrin, was apparently

disproved by Hammarsten (1896, 1899). This author found that a calcium free serum added to a calcium free plasma or a calcium free solution of fibrinogen causes clotting, and that in the fibrin formed calcium is present in such minute traces (0.005 per cent), as to preclude the possibility that it forms part of the fibrin molecule. Hammarsten's experiments have been repeated by Eagle (1935b) who obtained fibrin in which calcium was present only in the proportion of 1 part to 160,000 of protein. If the fibrin is considered as a calcium fibrinogen compound one must assume for the fibrinogen a molecular weight in excess of 1,000,000. Loucks and Scott (1930) while agreeing that fibrin is not a calcium compound assert that calcium is an essential constituent of thrombin since addition of oxalate to thrombin solutions destroys their activity. This result, however, is contradicted by Eagle. In his thrombin solutions the calcium present could be precipitated quantitatively without affecting their coagulating properties. This result is supported by the experiments of Kastl (1934) who finds that an active thrombin may be prepared, by Schmidt's method of alcohol precipitation, from serum which had been previously completely decalcified. That thrombin is not a calcium compound is indicated also by the fact that prothrombin, in the absence of calcium, may be activated to thrombin by other reagents, such as chloroform or alcohol (Howell, 1910; Cekada, 1926) or, indeed, under certain conditions may pass over to thrombin spontaneously. Mellanby (1933) reports that when his preparations of prothrombin were left in moist condition for twenty-four hours they were converted to thrombin, and the author has had the same experience with specimens of prothrombin prepared by his method of acetone precipitation from oxalated plasma. Whatever may be the change undergone by prothrombin in its conversion to thrombin it seems to be true that this change can take place in the absence of calcium, although this latter substance enormously accelerates the process. One would infer, therefore, that the calcium plays the rôle of a catalyser in accelerating a reaction that can take place spontaneously in its absence. This view, however, is not accepted by all the workers in the subject. In the most recent theories of coagulation, those of Fuchs and of Fischer, which will be referred to later, it is assumed that thrombin is a compound of prothrombin, calcium and a lipoid factor, but it may be said that in neither case is direct chemical evidence offered to prove the presence of calcium as an essential constituent of thrombin, although their work like that of others demonstrates that calcium plays a rôle in the production of thrombin.

Under normal conditions of the circulation the action of calcium alone is not sufficient to convert prothrombin to thrombin. This is apparent from the fact that in circulating blood both prothrombin and calcium are present, but no thrombin is formed. The additional factor required is the so-called tissue-factor furnished by the cellular elements and particularly by the blood-platelets. It has long been known that from the tissues or from the platelets a substance may be extracted which greatly facilitates coagulation. From the fresh tissue it may be extracted readily by water or saline solutions and from the dried tissue by means of alcohol. In general the aqueous extracts are more potent than the alcoholic extracts, and the former are thermolabile while the latter are not affected by boiling. There is, however, general agreement that the active constituent is the same in both forms of extracts, although present in a more reactive form in the extracts of fresh tissue. According to its mode of preparation and its supposed manner of action this tissue-factor has been given various names such as zymoplastic substance (Schmidt), thrombokinase (Morawitz), cytozyme (Bordet), thromboplastin or thromboplastic substance (Nolf, Howell), cephalin protein compound or cytozymphosphatid (Howell, Pickering, Fuchs).

While there is general agreement that in normal coagulation this factor coöperates with the calcium in the production of thrombin there is a difference of opinion in regard to its mode of action and its chemical nature. Morawitz believed that it functions as an organic activator or kinase, hence his designation of thrombokinase. According to Bordet the tissue-factor (cytozyme) combines directly, in varying proportions, with the serozyme (prothrombin) of the blood, in the presence of calcium, to form thrombin, and this view more specifically stated has been adopted by Fuchs, and by Fischer, who consider thrombin as a calcium lipoid protein compound. Howell has proposed a different explanation of its action. On the belief that prothrombin in the blood is protected by union with an inhibiting substance, antiprothrombin or heparin, he interprets the action of the tissue factor as a combination with the antiprothrombin or heparin whereby the prothrombin is liberated. He is supported in this view in part by Fuchs who believes that the action of the tissue factor is twofold, that it neutralizes the antiprothrombin and also combines with the liberated prothrombin. According to Pickering, and also Hekma, the chief action of the tissue factor is to cause the dissociation of the colloidal complex of prothrombin, fibrinogen and blood globulin and albumin which exists in the circulating blood.

Nature of the tissue factor. Owing to the fact that this factor may be

extracted from tissues by alcohol or ether and that in this form it is thermostable and shows the presence of phosphorus the earlier workers (Schmidt, Wooldridge, Bordet et al.) assumed that it is a substance of the nature of lecithin.

Howell (1912) called attention to the fact that as it occurs in the tissues it is practically insoluble in cold alcohol and soluble with difficulty in hot alcohol. He showed, as Wooldridge had done before, that egg lecithin has no favoring effect upon coagulation, and that purified lecithin from other sources is equally ineffective. He suggested that the active element in tissue extracts is a phospholipid of the nature of cephalin. Crude cephalin from the brain was found to have a marked thromboplastic action. Later in the same laboratory McLean (1917) tested the effect of purified preparations of cephalin from the liver, heart and brain and verified its influence upon coagulation. It was found that preparations of cephalin gradually lost their action upon standing in contact with the air, and since the same result followed when a purified preparation, furnished by Levene and West (1916), was reduced by the action of nascent hydrogen, it was assumed that the action of cephalin on coagulation is due to the unsaturated fatty acids in its molecules. The gradual loss of potency on standing was referred to a change in this condition from oxidation, and in accordance with this view it was found that when kept in vacuo in sealed tubes the potency was retained for long periods. This distinction between lecithin and cephalin was confirmed later by Gratia, working in Levene's laboratory (1922). He found that lecithin, "regardless of its form or origin" has no thromboplastic property, even in certain forms which contain unsaturated fatty acid groups. Cephalin on the other hand in undecomposed form exerts a decisive effect upon rate of clotting and this influence is detectable even in extreme dilutions. Similar results have been reported by Wadsworth, Maltaner and Maltaner (1927–1931), who likewise found that lecithin preparations free from cephalin have no effect upon coagulation, whereas specimens of pure cephalin have a high degree of activity.

In consequence of this work and its confirmation by other observers it has become generally accepted, perhaps, that cephalin in some form or combination constitutes the active element in tissue extracts which accelerates or initiates the act of clotting, and which specifically is concerned in the activation of prothrombin to thrombin. It has been assumed that in the tissues the cephalin exists in some combination, possibly a compound with protein, which is more potent than cephalin itself and which is thermolabile, and it is further assumed that such a

complex exists in the blood-platelets and accounts for their influence upon coagulation when they disintegrate in shed blood, but these assumptions are not based upon actual chemical determination or isolation of the substance in question. Fischer (1934), in contradiction to the results of Gratia and of Wadsworth, Maltaner and Maltaner claims that the purer the preparation of cephalin the less is its effect on coagulation. He believes that the thromboplastic influence exhibited by ordinary preparations is due either to some unknown impurity or to a complex of cephalin and cerebroside. More recently Charles, Fisher and Scott (1934) report that they have been able to isolate from the lungs, in crude form, a strong thromboplastic substance which, while a lipoid related to the fatty acids, is not cephalin. They designate this substance as an antiheparin. It is thermostable, resistant to oxidizing agents and soluble in fat solvents, especially petroleum ether. Its chemical nature is not known.

There can be no question that cephalin as usually prepared has a marked and perhaps unique action in facilitating clotting. Whether or not this result is due to cephalin itself, as seems most probable, or to some unknown material in combination with it, or present as an impurity, must be determined by future work. The undoubted fact that cephalin when freshly prepared is effective, but loses this property gradually on standing, is suggestive of the presence of some unknown factor, but, on the other hand, if the results obtained by McLean are corroborated this loss in potency may be adequately explained by changes within the cephalin molecule itself. It should be stated, however, that lecithin like cephalin contains or may contain unsaturated fatty acids. Comparison of the formulas assigned to lecithin and cephalin gives no indication as to what characteristic or grouping in the cephalin molecule may be called upon to explain the difference in the reaction of the two phospholipids in respect to blood coagulation. It seems probable that several lecithins and cephalins are present in animal tissues, differing from one another in the nature of the fatty acid groups in the molecule. According to Schmitz and Koch (1930) cephalin makes up from 25 to 30 per cent of the total phospholipid content of the blood plasma in most animals.

For the sake of clearness some of the divergent views regarding the nature of the tissue factor and its rôle in coagulation may be summarized briefly, as follows:

Morawitz—The active constituent functions as a kinase or co-enzyme which in connection with calcium converts prothrombin to thrombin.

Bordet—The active constituent, a phospholipid (lecithin) compound designated as cytozyme, unites directly with prothrombin (serozyme) to form thrombin.

Howell—The active constituent, a cephalin compound, combines with the antiprothrombin (heparin) of the prothrombin-antiprothrombin complex existing in circulating blood and liberates the prothrombin so that it may be activated to thrombin by the calcium of the blood.

Pickering—The active constituent, a protein phospholipid compound, causes dissociation of the complex of prothrombin with the fibrinogen and other proteins of the blood liberating the prothrombin in a form in which it can be activated to thrombin.

Mills—The active constituent, a protein cephalin compound, unites with calcium and fibrinogen to form fibrin.

Fuchs—The tissue factor consists of two constituents—one a cephalin compound which combines with or neutralizes antiprothrombin liberating the prothrombin, which is then activated to thrombin by the combined action of calcium and the cephalin compound; the other a prothrombin, similar in its property to blood prothrombin, and exerting the same effect, when activated, upon the blood fibrinogen.

Fischer—The active constituent is a lipoid complex of alcohol soluble cephalin and a cerebroside. This combines with prothrombin and calcium to form thrombin which is defined as a calcium containing lipoprotein.

The blood platelets. The most positive contribution to our knowledge of coagulation since the work of Alexander Schmidt was the discovery of the existence and reactions of the blood-platelets by Hayem and Bizzozero. Schmidt and his contemporaries assumed that the thrombin formed in shed blood arose from the disintegration of the leucocytes. The large granular masses observed in newly shed blood were supposed to originate from the fusion of broken down leucocytes. Hayem demonstrated the existence of the platelets in blood and described as one of their most striking properties the rapidity with which they agglutinate and disintegrate in blood removed from its normal environment. Bizzozero gave data to prove, contrary to the prevailing opinion of his day, that the leucocytes of the blood undergo little or no change during this period of clotting. It may be said that the definite and concordant results reported by Bizzozero (1882, 1883), and by Hayem (1877–1882) showed conclusively that the disintegration of the platelets is a preliminary step in the clotting of mammalian blood. They demonstrated,

moreover, that in the production of a thrombus the agglutination of the platelets is the initial phenomenon. On the basis of their work it has been generally believed that the disintegration of the platelets furnishes something to the plasma that breaks down the existing equilibrium and precipitates the act of clotting. In support of this view is the fact, noted first by both Hayem and Bizzozero and since confirmed by many observers, that anticoagulant substances, such as hirudin, heparin, oxalates, citrates, neutral salts, etc., which prevent coagulation also preserve the platelets from destruction. So also in hemophilic blood the platelets remain unchanged as long as the blood is fluid, and coagulation when it begins is accompanied and apparently preceded by platelet agglutination.

There are some considerations, however, which raise the question as to whether the agglutination of the platelets is really the initial act. There is a possibility that a change in some constituent of the plasma may be responsible for the alteration in the condition of the platelets. Deetjen (1909) has noted one exception to the effect of anticoagulants upon the platelets. In blood made incoagulable by the addition of a manganese salt, the platelets go to pieces, although more slowly than usual. On the other hand addition of certain organic peroxides to the blood preserves the platelets without preventing coagulation. He observed that platelets which had been treated for 10 to 15 minutes with hirudin were subsequently resistant to the action of alkali, calcium or serum, but underwent disintegration when mixed with plasma. Similarly it may be shown that platelets in oxalated or heparinized blood that have been washed free from plasma by salt solution show no tendency to agglutinate and indeed exhibit unusual stability. Whether, therefore, the initial step in coagulation is the destruction of the platelet, owing to contact with a foreign surface, as is usually believed, or whether some unknown change in the plasma brings about agglutination of the platelets is a question that, perhaps, is still open to discussion. However that may be there is no doubt that the disintegration of the platelets furnishes material that accelerates the clotting and probably is essential in the chemical changes connected with the activation of prothrombin to thrombin. Extracts of platelets, like extracts of tissues in general, but, perhaps, even more effectively, hasten the clotting of blood or initiate clotting in bloods that normally are incoagulable or clot very slowly, such as peptonized or heparinized blood or the slow clotting blood of the hemophilic.

The precise chemical nature of the material liberated by the disintegrating platelets is not known. No thorough study of the chemistry of these structures has been made in recent years. It has been shown that in addition to their protein constituents they contain lipoid material. Pekelharing, Lilienfeld and other of the older workers claim to have obtained from them a nucleoalbumin of importance to clotting, but it seems evident that their work should be repeated with more modern methods. It is stated that the ash of the platelets shows the presence of phosphorus, iron, calcium and sulphur. There is experimental evidence that their lipoid material is in part at least a phospholipid which is assumed by some workers to be a lecithin compound and by others, for reasons given above, to be a cephalin body. Haurowitz and Sládek (1928) report a comparative analysis of the composition of the platelets and the leucocytes of horse's blood, as follows:

	Thrombocytes *per cent*	Leucocytes *per cent*
Protein	71	69
Lipoid	12	22
Cholesterol	1.7	7.4
Ash	5.5	1.84
Ca	0.074	0.04

According to most observers (Morawitz, 1904; Bayne-Jones, 1912; Howell, 1914; Fuchs, 1928) the platelets contain prothrombin and are in fact the source of the blood-prothrombin. Bordet and others, however, deny that this is the case. On the whole the general conception of the relation of the blood platelets to coagulation is that in their rapid disintegration in shed blood they furnish to the plasma both prothrombin and the tissue factor, the thromboplastic material essential in one way or another to the production of thrombin.

Heparin (*antiprothrombin*). Heparin is the name given by Howell and Holt (1918) to an anticoagulant extracted from the dog's liver. At first thought to be a phospholipid further research demonstrated that the purified product is phosphorus free. Heparin is found in largest quantity in the liver of the dog, but according to Charles and Scott (1933), it is widely distributed in the animal body. In terms of the heparin unit, which is defined as the amount of heparin required to prevent the coagulation of 1 cc. of cat's blood for 24 hours, they give the following figures for its relative concentration in the different tissues.

Tissue	*Heparin units per kilogram*
Ox liver	1900
Ox liver	1900
Ox spleen	700
Ox heart	380
Ox blood	60
Ox thymus	35
Ox blood-serum	—
Ox lung	2200
Ox muscle	1900
Hog liver	1700
Dog liver	4400

The largest supply, according to this table, occurs in the liver, lungs and muscles. Confirming previous findings of Howell these authors state that the heparin is present in small concentration in full blood (blood plasma) but does not occur or, at least, cannot be detected in blood-serum.

The chemical nature of heparin has been investigated by several observers but the results reported are not in agreement. As purified by Howell's method (1928) it is described as an amorphous water soluble substance, containing neither phosphorus nor nitrogen, and yielding on analysis C 20.5 per cent, H 3.75 per cent, ash 37 per cent. It gives the Molisch reaction with alpha naphthol indicating the presence of a carbohydrate group. Heparin itself causes no reduction of copper salts, but after hydrolysis with dilute acid gives a positive reaction with Fehling's solution. Since it gives a glycuronic reaction with naphtha resorcinol and with orcinol (Bial's reagent) this author considers that it is either a paired glycuronate or a condensation product of glycuronic acid.

The ash of the purified product gives a reaction for calcium and for sulphuric acid, and when the heparin is treated with nitric acid (S. G. 1.15) and concentrated on the water bath crystals of calcium sulphate are deposited, hence it is assumed that in the purified product, as obtained by this method, both calcium and sulphuric acid are present. Schmitz and Fischer (1933) making use of the precipitate obtained with lead acetate were able to isolate what they regard as pure heparin in the form of a white, water soluble, nitrogen free powder which on analysis gave 34.6 per cent C., and 6.38 per cent H. They assign to the compound the formula $C_{18}H_{32}O_{17}6H_2O$. By treatment with brucin sulphate they obtained the brucin salt in crystalline form. Positive reactions with naphtharesorcinol and orcinol indicated the presence of a uronic

acid grouping. They consider heparin as a carbohydrate compound which contains one free carboxyl group due to the presence of the uronic acid and which acts, therefore, as a monobasic acid.

Charles and Scott (1933) by special methods of treatment, including the use of cadmium chloride to precipitate some of the impurities of the crude product, obtained a final substance of quite extraordinary potency, assaying as high as 400 to 500 units per milligram. This highly purified product was obtained in the form of a neutral substance, as a sodium compound and as a hydrochloride, the last named in crystalline form. Analysis gave the following figures.

	C	H	N	*Ash*
Free substance	22.01	3.70	2.7	33.27
Sodium salt	20.08	3.57	1.9	38.78
Hydrochloride	23.80	3.96	2.74	25.30

They believe that heparin contains both a free carboxyl and a free NH_2 group and reacts, therefore, like an amphoteric compound. The presence of a carbohydrate group was indicated by a positive Molisch reaction, but their product gave entirely negative reactions with naphtharesorcinol and orcinol, excluding, therefore, the view that a uronic grouping is present. In a preliminary communication Jorpes (1935) reports that he has obtained from preparations of purified heparin a hexuronic acid to the amount of 17 to 19 per cent, and a hexoseamine to the amount of 10 to 14 per cent. He believes that these constituents are combined in the proportion of one molecule of hexuronic acid to one molecule of hexoseamine. This supposition is borne out by a determination of the acetyl present in the hexoseamine. His analyses indicated a ratio of one molecule of acetic acid to one molecule of hexuronic acid. Sulphur was found to the amount of 11.2 per cent of which about 7.9 per cent is probably in organic combination. He states that the large amount of ash, 40 per cent, consisted almost entirely of magnesium sulphate. The author believes that heparin is a chondroitin polysulphuric acid. The usual chondroitin sulphuric acid has no anticoagulant action. It seems evident that further study is required to determine the precise chemical nature of heparin and the molecular grouping which controls its anticoagulant effect.

Heparin in crude form with a potency of 5 to 15 or 20 units per milligram is now obtainable commercially. It has proved to be a useful agent in experimental work in which it is necessary to prevent clotting of the blood, since it causes no injury to the animal when injected into the circulation. The normal presence of heparin in blood-plasma has

been demonstrated by several observers (Howell, Fuchs, Charles and Scott) and Howell has shown that in the blood of peptonized dogs the amount is greatly increased, thus explaining its incoagulability. In his theory of coagulation Howell has assumed that the fluidity of the circulating blood is due to the presence of heparin which by combining with the prothrombin protects it from being activated. When blood is shed this combination is broken down by the tissue factor (protein cephalin complex) added to the blood, which combines with the heparin and liberates the prothrombin. The experimental results offered in support of this theory have been confirmed by Fuchs and others, but are denied and otherwise explained by Mellanby (1934). This author was not able to obtain any evidence that heparin combines with prothrombin. Heparin added to a mixture of prothrombin, calcium and kinase (tissue extract) did not prevent the activation of the prothrombin, as tested subsequently by the clotting effect of the mixture on dialyzed oxalated plasma. Howell, on the contrary, reports that addition of heparin in sufficient amounts to a mixture of prothrombin, calcium and tissue factor completely prevents the formation of thrombin, as determined by a test solution of fibrinogen. Opposite results of this character, so common in the literature of coagulation, are probably due to differences in the reagents used. Mellanby's prothrombin was prepared by a different method from that used by Howell, and the test solution for the presence of thrombin was in one case a purified solution of fibrinogen and in the other an oxalated plasma which had been dialyzed for 6 hours against distilled water to remove the salts.

One of the properties of heparin which has been emphasized by Howell is that this compound has no inhibiting effect upon the reaction between thrombin and fibrinogen, when purified preparations of these reagents are used. Heparin, therefore, unlike hirudin does not react with thrombin and in this sense is not an antithrombin but an anti-prothrombin. Other authors state that the heparin has a real antithrombin action, but in most if not in all such cases the criticism may be made that the reagents used were not adequately purified. Mellanby, for example, used a fibrinogen that had been prepared by a single precipitation from plasma. There can be little doubt that such a preparation was contaminated with prothrombin.

When heparin is added to plasma or serum it has the characteristic that it causes the production in these liquids of a true antithrombin by a reaction with some unknown thermolabile constituent of the blood. If the plasma is heated to 70°C. this reaction does not take place. Mel-

lanby finds an explanation of this increased antithrombic power in a combination or reaction of some kind between heparin and the salts of the blood. When the salts are removed by dialysis heparin ceases to give this reaction. His explanation fails to account for the fact that heating the plasma to 70°C. destroys the reaction. Mellanby's theory of the anticoagulant action of heparin seems to be that in the presence of inorganic salts it acquires the property of an antithrombin, while Howell claims that heparin has the specific property of combining with the precursor of thrombin, the prothrombin. Outside the direct experimental evidence offered in support of this view it serves to explain such observations as that reported by Fuld and Spiro (1904) that blood serum, containing thrombin, will cause the clotting of peptonized plasma, while an equal amount of full blood, containing only prothrombin, is ineffective.

Waldschmidt-Leitz (1929), on theoretical grounds, has suggested as a third possibility that the heparin may exert its anticoagulant action by combining with and neutralizing the tissue factor (protein cephalin complex). This view is based on the fact that trypsin destroys the anticoagulant action of hirudin but not that of heparin. This difference is taken as an indication that the heparin, unlike the hirudin, is not a proteolytic substrate and cannot, therefore, react with thrombin which is regarded as a proteolytic enzyme of the nature of trypsin.

In view of the marked anticoagulant activity of heparin and its normal presence in the blood the suggestion naturally occurs that variations in coagulability, especially such a condition as hemophilia, may find their explanation in changes in concentration of the heparin. For hemophilia the statement has been made by Fuchs that there is an increase of heparin in the blood, but he gives no direct proof that this is actually the case. There is no method for quantitative estimation of heparin that is adequate for the detection of small differences in concentration, but Howell (1926) and Evans and Howell (1931) were unable to find any significant difference in this respect between normal and hemophilic blood.

Antithrombin and metathrombin. The term antithrombin has been used at times to designate any anticoagulant substance or effect discovered in the blood or tissues, but in a strict sense it is limited to a substance of unknown chemical nature which occurs normally in small concentration, in blood plasma and serum and lymph. It has the property of combining with or neutralizing active thrombin. Whether or not it acts similarly with the prothrombin of plasma has not been

determined. It was first definitely studied by Morawitz and has since been investigated by many workers. Howell used a simple method for determining its relative concentration and he and his co-workers have published data in regard to its variations in blood-plasma under normal and pathological conditions. As stated above he finds that a characteristic property of heparin is that when added to plasma or serum it causes a marked increase in the amount of antithrombin.

Metathrombin is the name given by Morawitz to an inactive or modified form of thrombin, altogether different from prothrombin, which develops progressively in serum after the clotting of the blood. So far as can be determined it is not present in the plasma of the circulating blood. The existence of such a substance in the serum was known to Schmidt who devised a successful method for reactivating it to thrombin or, at least, for obtaining active thrombin from it. This method, known as the method of alkali activation consists in treating the serum with a N/10 solution of sodium hydroxide for 15 minutes and then neutralizing with N/10 solution of hydrochloric acid. The reaction takes place in the absence of calcium. The development of an active thrombin is shown by the coagulating effect of the serum, after treatment, upon a solution of fibrinogen or oxalated plasma. The researches of Weymouth (1913), Gasser (1917) and Rich (1917) make it probable that metathrombin is a compound of thrombin and antithrombin. The reaction is an irreversible one. The process of alkali activation liberates thrombin by destroying the antithrombin. It seems possible that the formation of metathrombin plays a protective rôle in the normal circulation by inactivating any free thrombin that may be formed under normal or abnormal conditions. The many interesting facts and speculations regarding the nature, relations and functional significance of metathrombin and antithrombin are treated in detail in Wöhlisch's review of 1929. So far as the author knows no new investigations of importance have appeared since that time.

Theories of coagulation. So far as the non-thrombin theories of coagulation are concerned, the theories of Wooldridge, Nolf, Mills, Stuber and Lang, enough, perhaps, has been said already to indicate their salient features. The more orthodox theories have also been referred to in connection with the discussion of the factors of coagulation, but they may be summarized very briefly, at the risk of repetition, to bring out the relations of the older and the newer points of view. These theories are based upon four fundamental facts which were discovered early in the modern history of the subject, namely,

1. Coagulation is caused by the development in shed blood of a ferment like body, thrombin, which in the circulating blood exists in an inactive form, prothrombin. (Alexander Schmidt, 1872.)

2. Fibrin is formed by a reaction between thrombin and fibrinogen (Hammarsten, 1880).

3. Calcium is essential in normal clotting (Arthus and Pagès, 1890).

4. The tissue cells furnish a constituent which is concerned in the formation of thrombin (Alexander Schmidt, 1893).

These facts were first combined in what might be called a successful theory, that is one that enjoyed a wide acceptance, by Morawitz (1904), and practically simultaneously, in somewhat different form, by Fuld and Spiro (1904).

The theory of Morawitz (1904). The theory assumes that the factor furnished by the tissue cells is an organic kinase and together with the calcium activates the prothrombin to thrombin, which then reacts with the fibrinogen. In the circulating blood prothrombin, calcium and fibrinogen are present but the organic activator, to which he gave the name thrombokinase, is lacking, hence the prothrombin remains in inactive form. When blood is shed the platelets disintegrate rapidly and furnish thrombokinase. The thrombokinase normally derived from the platelets may be furnished by any of the tissues, especially if wounded. His theory may be expressed by the following simple schema:

Prothrombin (thrombogen) + Ca + Thrombokinase = Thrombin

Thrombin + Fibrinogen = Fibrin

The characteristic feature of the theory is the hypothesis that the tissue factor acts as a kinase, thrombokinase.

The theory of Bordet (1912). Bordet accepts the view that fibrin is formed by a reaction between thrombin and fibrinogen, but he gives a different explanation of the origin of the thrombin. The factor furnished by the platelets, to which he gives the name cytozyme, is not a kinase but a lipoid (lecithin) compound which, in the presence of calcium, unites chemically with the prothrombin of the blood, designated by him as serozyme, to form thrombin. Bordet's serozyme does not correspond exactly with the prothrombin of Schmidt or the thrombogen of Morawitz, since in its reactive form it is found only in shed blood. In the circulating blood it is present in a non-reactive form, a prosero-zyme, owing possibly to combination with an inhibiting or protective substance. When the blood is shed contact with a foreign surface, i.e., a wetable surface, suffices, when calcium is present, because of some

physico-chemical reaction, to alter the proserozyme to serozyme, so that, in the presence of calcium it can unite with the cytozyme which is furnished by the disintegrating platelets. Three successive reactions occur in clotting which may be represented schematically as follows:

1. Proserozyme converted to serozyme
2. $\left.\begin{matrix}\text{Serozyme (prothrombin)}\\ \text{Cytozyme (tissue factor)}\end{matrix}\right\}$ in the presence of Ca = thrombin
3. Thrombin + fibrinogen = fibrin.

In Bordet's theory, therefore, two new hypotheses are made for which evidence of an indirect kind is furnished in his numerous papers. First, that thrombin is a compound or combination of serozyme and cytozyme, the former being considered as a protein and the latter as a lecithin compound. Second, that the initial step in clotting is a physical contact reaction which changes proserozyme to serozyme.

The theory of Howell (1918, 1925). The central feature of this theory is the hypothesis that in circulating blood there is an inhibiting substance which prevents coagulation. While a number of workers, Morawitz, for example, have considered the possibility or probability of the presence of an inhibiting substance, designated usually as an antithrombin, it has not been taken into account specifically in their theories of the coagulation process. They have explained the fluidity of the blood as due to the absence of some essential factor of coagulation. The inhibiting or protective substance in Howell's theory is heparin which occurs normally in blood and which is considered to act as an antiprothrombin rather than as an antithrombin. In the circulating blood the heparin is combined with prothrombin to form a prothrombin—antiprothrombin complex. When the blood is shed the tissue-factor liberated from the platelets (or wounded tissue-cells), which he believes to be a cephalin compound, combines with the heparin and sets free the prothrombin. The prothrombin is then activated to thrombin by the calcium of the blood. This author believes that the disintegration of the platelets furnishes not only the tissue factor but also additional prothrombin. The plasma of the circulating blood is assumed to contain in solution all of the necessary factors in the clotting reaction, namely, fibrinogen, prothrombin and calcium, but the reaction is prevented by the presence of heparin. In this theory three successive reactions take place:

1. Prothrombin-heparin + tissue factor (cephalin) = free prothrombin
2. Prothrombin + Ca = thrombin
3. Thrombin + Fibrinogen = fibrin.

As compared with previous theories three new hypotheses are made.

First, the existence in blood of an inhibiting substance (heparin) responsible for maintaining its fluidity.

Second, the active constituent of the tissue factor is a cephalin rather than a lecithin compound.

Third, prothrombin is activated to thrombin by calcium without the coöperation of an organic kinase.

The theory of Pickering (1925, 1928). The main feature of this theory is the hypothesis, first proposed by Mellanby (1909), that the prothrombin in circulating blood exists in physico-chemical combination with fibrinogen. In the latest form of his theory he describes this combination as a balanced complex of the less stable (prothrombin, fibrinogen) and the more stable (serum globulin and serum albumin) blood proteins. Prothrombin is thus protected by its union with the more stable colloidal compounds of the blood. Whatever causes dissociation of this colloidal complex sets free the prothrombin so that, in the presence of calcium, it becomes activated to thrombin. The complex has only a limited resistance to disturbing influences. It undergoes dissociation whenever the blood comes into actual contact with a foreign surface, that is to say, with a surface which the blood can wet, and it is dissociated also by the action of tissue extracts. Actual clotting, the transition from a sol to a gel condition, may take place under ordinary circumstances in three ways: 1. By the physical effect of contact with a wetable surface, without the intervention of cell constituents. The contact suffices to break down the colloidal complex and to liberate the prothrombin under conditions which permit its activation to thrombin. 2. By the action of the tissue factor, defined as a protein cephalin compound, liberated in the disintegration of the blood-platelets. The tissue factor causes dissociation of the colloidal complex and, in the presence of calcium unites with the prothrombin to form thrombin which then reacts with fibrinogen to form fibrin. This latter reaction in some way sets free the remainder of the prothrombin causing a progressive increase in the speed of clotting. 3. By a direct reaction between tissue juice and plasma fibrinogen, in accordance with the views proposed by Mills. In the clotting of blood in wounds it is assumed that "the second and third modes of gel formation apparently take place simultaneously."

The theory of Fuchs (1929, 1933). In this theory the existence and action of five different factors are considered, namely, fibrinogen, calcium, prothrombin, antiprothrombin (heparin) and the tissue element or factor which, following Bordet, he designates as cytozyme or cytozymephosphatid, although he accepts the view that the active constitu-

ent is a cephalin compound. These factors are all present in the circulating blood, but the prothrombin is protected from being activated by combination with antiprothrombin, this prothrombin-antithrombin complex representing the protected form of prothrombin which Bordet designated by the term proserozyme. The cytozyme phosphatid in the plasma is also not in free form, but exists as an inactive protein complex. Active thrombin he believes with Bordet is formed by combination between prothrombin and cytozyme in the presence of calcium. The distinctive feature of Fuchs' theory is that in ordinary clotting prothrombin is furnished or may be furnished from three sources. First, from the disintegrating platelets. In the platelets prothrombin and cytozyme are present preformed in optimal proportions to produce thrombin, but the reaction is prevented by the absence of calcium. On disintegration of the platelets calcium is supplied by the plasma and active thrombin is produced which then reacts with the fibrinogen to form fibrin. The author believes that this is the quickest source of thrombin in shed blood. Second, from the plasma itself. The disintegration of the platelets furnishes an excess of cytozyme and some of this reacts with the prothrombin-antiprothrombin complex of the normal plasma, and indeed exerts two effects. It combines with the antiprothrombin and sets the prothrombin free, and secondly, it unites with the freed prothrombin in the presence of calcium to form thrombin. Third, from the wounded tissue elements. Fuchs holds that in muscular and other tissues prothrombin is a normal constituent, together with cytozyme and antiprothrombin. From contact with wounded tissue blood, therefore, gets a supply of prothrombin which combines with the cytozyme and calcium to form thrombin. In ordinary hemorrhage these three processes go on simultaneously, but, as stated, it is the platelet prothrombin which first becomes activated and starts the reaction with fibrinogen.

While the prothrombin of muscle is identical with that of blood and may be substituted for it in blood coagulation, the normal function of the muscle prothrombin is to initiate the clotting of muscle plasma. On the theory that muscle contraction and relaxation are dependent upon a reversible coagulation of muscle proteins muscle prothrombin is given a leading part in the production of muscle tonus and contraction. In addition Fuchs believes that prothrombin of blood is identical with the middle piece of the complement. The numerous researches undertaken by him and his collaborators to establish this identity seek to show that all conditions that prevent blood-clotting by keeping the

prothrombin in inactive form, such as the action of heparin and hirudin, serve at the same time to prevent the complement activity of the plasma. So also when prothrombin is removed from plasma by adsorbents, such as magnesium hydroxide, complement activity is lost, and when complement is removed by the specific adsorptive activity of sensitized corpuscles spontaneous coagulability disappears. Prothrombin, therefore, he regards as a non-specific substance which by different processes may be converted into one or another of three specific ferments, namely, blood thrombin, muscle thrombin and complement or hemolyzing substance, which are not interchangeable.

The theory of Fischer (1933, 1934).* Prothrombin according to this author is a protein of a globulin nature with its isoelectric point on the acid side, pH 5.3. It occurs in the tissues generally as well as in the blood plasma, and in both blood and tissues it is converted to thrombin by a reaction with calcium and the tissue-factor. The tissue factor, for which he uses Morawitz's term thrombokinase, is a lipoid complex of an alcohol soluble form of cephalin and a cerebroside. Cephalin alone is ineffective (Fischer and Hecht, 1934). In forming thrombin the thrombokinase unites with the prothrombin which it modifies or denatures. Thrombin is defined, therefore, as a calcium containing lipoprotein. In reacting with prothrombin thrombokinase exerts two distinct effects. First, it modifies or denatures the prothrombin protein so that it becomes isoelectric near the neutral point. This effect is not specific to thrombokinase since the same result may be obtained by the action of acids and of other organic compounds. It is a change, however, that is essential to clotting since thrombin is effective in clotting only at its isoelectric point. Second, the thrombokinase exerts a specific effect of some kind upon the prothrombin which makes possible the coupling of the thrombin complex with fibrinogen.

The effect of heparin upon clotting (Fischer and Schmitz, 1933) is explained on the theory that it unites with prothrombin, changing its isoelectric point further toward the acid side and preventing thrombokinase from exerting upon it the denaturing action, the non-specific action, which is essential in changing prothrombin protein to thrombin protein. Once the prothrombin has been denatured by thrombokinase, acids, etc., heparin has no anticoagulant action.

* Fischer gives a summary, in English, of his studies on coagulation (The Japanese Journ. of Exp. Med. **13**: 223, 1935) which was received too late to be used in this review.

SUMMARY

In view of the numerous discordant theories of coagulation that exist to-day it is difficult or impossible to determine what positive increase in knowledge has resulted from the work of recent years. One might have hoped that the application of the principles of physical and colloidal chemistry to the great mass of data that has been accumulating for years in this field would have brought into the subject certain unifying conceptions that would tend to remove some of the confusion that exists, but as a matter of fact this expectation has not been fully realized, so far at least. The researches of Hekma, Stuber and Lang, Fischer and other workers who have used these modern methods of study have given us new points of view but points of view that in some respects are speculative and unconvincing, and that in large part seem to overlook actual observations put on record by previous investigators. In a liquid like the blood there can be no doubt that colloidal conditions influence the course of events and that physico-chemical relations play a rôle. It is, however, also evident that coagulation involves a series of chemical reactions whose nature may be discovered and demonstrated in such a way as to command general acceptance and to constitute a solid basis for further work. Unfortunately we have not as yet reached this vantage point. One difficulty, perhaps, that stands in the way is that the reagents which have been employed by different workers in studying these reactions have been lacking in uniformity. Partly from necessity these reagents have been usually mixtures of varying composition. As a test solution for the reaction of coagulation some workers have used solutions of fibrinogen, with or without the removal of known impurities, such as prothrombin; others have preferred to utilize for this purpose an oxalated plasma, dialyzed or undialyzed or a plasma deprived of spontaneous coagulability by some other device. So also for a coagulating agent preparations of thrombin or prothrombin of varying composition have been employed and the same is true for the so-called tissue factors. If heparin is brought into the series of experiments many have been satisfied to use the commercial product, mostly consisting of impurities of an unknown character, thereby introducing constituents which may influence the course of the reaction. Standard methods and reagents have not been developed and it is not surprising therefore that varying and contradictory results have been obtained. Perhaps no satisfactory progress toward uniformity of results is to be expected until the several reagents that enter into the process of coagulation, namely, fibrinogen,

thrombin and prothrombin and antiprothrombin, the tissue factor, etc., can be prepared in purified and standardized form.

At the end of his discussion of the *Physiology of Blood Coagulation* in his review of 1929, Wöhlisch sums up briefly what may be considered as established facts. The fundamental points are, in the first place, the acceptance of Hammarsten's division of the process of coagulation into two chief phases or stages,

1. The formation of thrombin from its precursors
2. The conversion of fibrinogen to fibrin under the influence of thrombin

and in the second place the statement that the necessary precursors of thrombin are prothrombin, calcium and a tissue element (thrombokinase cytozyme, etc.). In substance this represents the theory of coagulation as it was formulated by Morawitz in 1904 to 1905. It may be doubted whether we can go much farther than this at present in attempting to find a common ground acceptable to the majority of workers in the subject. It may be said that in most of the thrombin theories of coagulation that are current to-day it seems to be agreed that the precursors of thrombin, namely, prothrombin, calcium and the tissue factor, unite chemically to form thrombin. As against this view it may be noted that two workers (Mellanby, Howell) claim to have prepared an effective thrombin which gives no reaction for phosphorus and which, therefore, contains no phospholipid. One might add, possibly, that the trend of recent work tends to support the view that the active chemical constituent in tissue extracts is a phospholipid of the nature of cephalin rather than lecithin, and there is perhaps an increasing recognition of the fact that an inhibitory or protective factor of the nature of heparin may be concerned in maintaining the normal fluidity of the blood, but in regard to the details of the formation of thrombin and the nature of its reaction with fibrinogen there is still a wide and apparently constantly increasing divergence of opinion. One seeks in vain to reconcile these views or to reduce them to a common basis.

REFERENCES

References are given only to sources specifically quoted in the text. The complete bibliography of coagulation is very large. The older literature is given in the review by Morawitz (1905) and the more recent references in the reviews by Wöhlisch (1929), Fuchs (1930 and 1933), and Zunz (1934), as also in Pickering, *Blood Plasma in Health and Disease* (1928).

ARTHUS, M. AND C. PAGÈS. Arch. d. Physiol. norm. et path. **22**: 739, 1890.

BAYNE-JONES, S. Am. J. Physiol. **30**: 74, 1912.

BIZZOZERO, J. Virchow's Arch. **90**: 261, 1882.
Arch. ital. d. Biol. **3**: 94, 1883.
BOHR, C. Centralbl. f. Physiol. 261, 1888.
BONNE. Ueber das Fibrinferment, etc. Würzburg, 1889.
BORDET, J. Ann. Inst. Pasteur **34**: 561, 1921.
Johns Hopkins Hosp. Bull. **32**: 213, 1921.
BORDET, J. AND L. DELANGE. Ann. Inst. Pasteur **26**: 657, 737, 1912.
Ann. Inst. Pasteur **27**: 341, 1913.
BUCHANAN, A. London Med. Gaz. **1**: 617, 1845. (Reprinted in J. Physiol. **2**: 158, 1879.)
CEKADA, E. B. Am. J. Physiol. **78**: 512, 1926.
CHARLES, A. F. AND D. A. SCOTT. J. Biol. Chem. **102**: 425, 431, 437, 1933.
CHARLES, A. F., A. M. FISHER AND D. A. SCOTT. Tr. Roy. Soc. Canada **28**: Sec. V, 49, 1934.
DEETJEN, H. Ztschr. f. physiol. Chem. **63**: 1, 1909.
DEL BAERE, L. J. Biochem. Ztschr. **246**: 38, 1932.
DENIS, P. S. Mémoire sur le Sang. Paris, 1859.
DOYON, MOREL AND KAREFF. Compt. rend. soc. biol. 705, 1905.
DRINKER, C. K. AND K. R. DRINKER. Am. J. Physiol. **41**: 5, 1916.
EAGLE, H. J. Gen. Physiol. **18**: 531, 1935a.
Ibid. 547, 1935b.
EVANS, P. S. AND W. H. HOWELL. Am. J. Physiol. **98**: 131, 1931.
FISCHER, A. Biochem. Ztschr. **264**: 169, 178, 184, 1933.
Biochem. Ztschr. **269**: 115, 1934.
FISCHER, A. AND E. HECHT. Biochem. Ztschr. **269**: 115, 1934.
FISCHER, A. AND A. SCHMITZ. Biochem. Ztschr. **259**: 67, 1933.
FUCHS, H. J. Ztschr. Imm.-forsch. u. exp. Therap. **59**: 424, 1928.
Ergebn. d. inn. Med. **38**: 173, 1930.
Ergebn. d. Enzym forsch. **2**: 282, 1933.
FUCHS, H. J., M. VON FALKENHAUSEN AND E. HARTMANN. Ztschr. ges. exp. Med. **64**: 227, 1929.
FUCHS, H. J. AND M. VON FALKENHAUSEN. Ztschr. ges. exp. Med. **79**: 23, 1931.
FUCHS, H. J. AND Z. ZAKRZEWSKI. Klin. Wchnschr. October 20, 1934.
FULD, E. AND K. SPIRO. Hofmeister's Beiträge **5**: 174, 1904.
GAMGEE, A. J. Physiol. **2**: 145, 1879.
Physiological chemistry of animal body. London, 1880.
GASSER, H. S. Am. J. Physiol. **42**: 378, 1917.
GRATIA, A. AND P. A. LEVENE. J. Biol. Chem. **50**: 455, 1922.
HAMMARSTEN, O. Pflüger's Arch. **19**: 563, 1880.
Ibid. **30**: 437, 1883.
Ztschr. f. physiol. Chem. **22**: 333, 1896.
Ibid. **28**: 98, 1899.
HARTMANN, E. AND J. KÜHNAU. Ztschr. ges. exp. med. **73**: 720, 1930.
HAUROWITZ, F. AND J. SLÁDEK. Ztschr. f. physiol. Chem. **173**: 233, 1928.
HAYEM, G. Compt. rend. Acad. Sci. **135**: 58, 1878.
Union méd. 2 sém., 1882.
HEKMA, E. Biochem. Ztschr. **143**: 105, 1923.
Arch. Neerl. d. Physiol. **16**: 274, 1931.

HOWELL, W. H. Am. J. Physiol. **26**: 452, 1910.
Ibid. **31**: 1, 1912.
Ibid. **35**: 474, 1914.
Ibid. **40**: 526, 1916.
Proc. Inst. of Med. Chicago, 1925.
Am. J. Physiol. **77**: 680, 1926.
Bull. Johns Hopkins Hosp. **42**: 199, 1928.
HOWELL, W. H. AND E. HOLT. Am. J. Physiol. **47**: 328, 1918.
HUISKAMP, W. Ztschr. f. physiol. Chem. **44**: 182, 1905.
JONES, T. B. AND H. P. SMITH. Am. J. Physiol. **94**: 144, 1930.
JORPES, E. Die Naturwissenschaften, March 22, 1935.
KASTL, O. Biochem. Ztschr. **274**: 452, 1934.
KLINKE, K. Klin. Wchnschr. **10**: 869, 1931.
KRAUS, F. AND H. J. FUCHS. Ztschr. ges. exp. Med. **64**: 583, 1929.
KÜHNAU, J. AND MORGENSTERN. Die Naturwissensch., July 27, 1934.
LEVENE, P. A. AND WEST. J. Biol. Chem. **24**: 43, 52, 1916.
LOUCKS, M. M. AND F. H. SCOTT. Am. J. Physiol. **91**: 27, 1929.
MCLEAN, J. Am. J. Physiol. **43**: 586, 1917.
MAINZER, F. Biochem. Ztschr. **246**: 164, 1932.
MELLANBY, J. J. Physiol. **38**: 28, 1909.
Proc. Roy. Soc. B. **107**: 271, 1930.
Ibid. **113**: 93, 1933.
Ibid. **116**: 1, 1934.
MILLS, C. A. AND G. M. GUEST. Am. J. of Physiol. **57**: 395, 1921.
MILLS, C. A. AND A. P. MATHEWS. Arch. internat. Physiol. **24**: 73, 1924.
MORAWITZ, P. Hofmeister's Beitr. **5**: 174, 1904a.
Arch. f. Klin. Med. **79**: 215, 1904b.
Ergebn. d. Physiol. **4**: 368, 1905.
NOLF, P. Arch. internat. Physiol. **4**: 165, 1906.
Ergebn. inn. Med. u. Kinderheilk. **10**: 275, 1913.
For condensed statement, see PICKERING, Blood plasma in health and disease, 1928, p. 130.
PARTOS, A. Pflüger's Arch. **229**: 336, 1932.
PAVLOV, I. P. Arch. f. Physiol. p. 452, 1887.
PEKELHARING, C. A. Untersuchungen ü d. Fibrinferment. Amsterdam, 1892.
PICKERING, J. W. Brit. J. Exp. Biol. **2**: 397, 1925.
Blood plasma in health and disease. London, 1928.
RICH, A. Am. J. Physiol. **43**: 549, 1917.
SCHMIDT, A. Arch. f. Physiol. p. 545, 1861.
Pflüger's Arch. **6**: 445, 1872.
Zur Blutlehre. Leipzig, 1893.
Weitere Beiträge zur Blutlehre. Wiesbaden, 1895.
SCHMITZ, E. AND F. KOCH. Biochem. Ztschr. **223**: 257, 1930.
SCHMITZ, A. AND A. FISCHER. Ztschr. f. physiol. chem. **216**: 264, 274, 1933.
SCOTT, F. H. AND C. CHAMBERLAIN. Proc. Soc. Exp. Biol. and Med. **31**: 1054, 1934.
SMITH, H. P., E. D. WARNER AND K. M. BRINKHOUS. Am. J. Physiol. **107**: 63, 1934.
STUBER, B. Kolloidztschr. **51**: 144, 1930.

STUBER, B. AND K. LANG. Physiologie und Pathologie der Blutgerinnung. Berlin and Vienna, 1930.
STUBER, B. AND F. FOCKE. Biochem. Ztsch. **154**: 77, 1924.
VINES, H. W. C. J. Physiol. **55**: 86, 287, 1921.
WADSWORTH, A., F. MALTANER AND E. MALTANER. Am. J. Physiol., **80**: 502, 1927.
Ibid. **91**: 423, 1930.
Ibid. **97**: 74, 1931.
WALDSCHMIDT-LEITZ, E., P. STADLER AND F. STEIGERWALDT. Ztschr. physiol. Chem. **183**: 39, 1929.
WEYMOUTH, F. W. Am. J. Physiol. **32**: 266, 1913.
WÖHLISCH, E. Ergebn. d. Physiol. **28**: 443, 1929.
WÖHLISCH, E. AND K. PASCHKIS. Ztschr. ges. exp. med. **40**: 121, 1924.
WOOLDRIDGE, J. C. Arch. f. Physiol. p. 397, 1886.
Beitr. z. Physiol. (Ludwig), 1887.
ZUNZ, E. Traité d. Physiol. (Roger et Binet) **7**: 189, 1934.

THE BIOLOGICAL SIGNIFICANCE OF COPPER AND ITS RELATION TO IRON METABOLISM

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Although copper as a metal has been known for centuries, the full realization of the importance of its compounds in biological activities has come only recently. The significance of this element has generally centered around its value as a constituent of specific compounds in certain animal species. It is only within the last decade that the universal distribution of copper in plants and animals has been given serious consideration. Investigators first attributed no definite function to its presence and generally assumed the occurrence to be accidental. The very recent work which especially emphasizes the value of copper in another definite animal phenomenon, namely, hemoglobin formation, gives a renewed interest in the earlier work on this element.

Discussions of the possible occurrence of copper in plants and animals can be traced in the literature for the past one hundred years. Miessner (1817) was probably the first worker to show that copper actually was a constituent of plants. Later Sarzeau (1830) found copper in numerous plants and determined the amount quantitatively. The work of Deschamps (1848) in the further study of vegetables revealed that a relationship existed between the copper in plants and that in the soil on which they were grown, while Chevreul (1868) in a more general statement suggested that copper was quite widely distributed in organic matter. Lehmann (1895) summarized the known facts regarding the distribution of copper at that time and added many results from his own analyses. In spite of the fact that much of the work reported at this early date is surprisingly complete, undoubtedly many of the figures are erroneous due to the use of faulty methods of analysis.

The later work of Maquenne and Demoussy (1920), Guerithault (1920), and Fleurent and Levi (1920) has established without question the universal distribution of copper in plant life. Maquenne and Demoussy found 3 to 40 parts per million of copper in all the plant materials they analyzed and suggested that copper must be an essential element in plant metabolism. The fact that it was found in the more active

organs of the plant; e.g., young shoots and leaves, naturally led them to conclude that copper in some way aided in the vital functions of the plant. Guerithault's figures ranged from 1 to 17 mgm. per kilo of fresh material and were highest for seeds. Fleurent and Levi, however, found the largest amount in the leaves. Guerithault, as well as Bertrand (1920), suggests that copper should be placed in the group of catalytic elements found in plants.

The fact that marine animals contain copper in considerable amounts is, of course, well known. Harless (1847) detected this element in Eledone and Helix pomatia, and showed that it did not exist as a free salt but in combination with blood proteins. Dhere (1915) has reviewed

TABLE 1

Copper content of bloods containing hemocyanin

GROUP	ANIMAL	Copper
		mgm. per 100 cc.
Mollusca Cephalopoda	Octopus vulgaris	23.5
	Sepia officinalis	23.7
Mollusca Gastropoda	Helix pomatia	6.5–7.5
Crustacea Decapoda	Astacus fluviatilis	7.0
	Palinurus vulgaris	9.5
	Homarus vulgaris	10.0
	Cancer pagurus	6.0
	Carcinus maenas	9.0
	Maia squinado	3.5
Crustacea stomatopoda	Squilla mantis	6.1

most completely the early investigations concerning the copper content of the blood of marine animals. Table 1, compiled from Dhere's data, shows the relative distribution of copper in the blood of these animals. If the copper in these organisms is obtained from the sea water there must be a tremendous concentration since sea water contains only about 10 mgm. of copper per cubic meter (Atkins, 1932).

Rose and Bodansky (1920) also present a table showing the animals in which copper was known to occur at that time and point out that the data thus far accumulated are chiefly confined to arthropods and mollusks. They made a quantitative study of copper in thirty-five marine animals, their work being confined mainly to fishes. Copper was found

to be present in all the fish studied with the exception of pigfish, the amount in each of the individual samples averaging about 2.5 mgm. per kilo. They point out that it is impossible to state whether the copper exists as hemocyanin or as other compounds of physiological importance. The amount of copper in the organisms examined frequently diminished as the animals ascended from the comparatively simple to the more complex; and when the fishes, which are provided with hemoglobin were reached, the copper persisted at a lower level.

Clams were found to contain very little, if any, copper while oysters were found to be exceedingly rich in this element, from 24 to 60 mgm. per kilo. The work of Willard (1908) and Hiltner and Wichmann (1919) had already shown that oysters were very rich in copper. These workers suggest that oysters may carry enough copper to give a greenish color and a metallic taste. Rose and Bodansky found the quantitative distribution of copper in the various organs of the oyster to be quite uniform, although the muscle fraction contained less than the other parts. The copper content of the shrimp and crab lies intermediate between that of the oyster and other fish. The largest portion of the copper is found in the protective covering of the crab. Two forms of coelenterates, jellyfish (Aurelia), and Portugese man-of-war (Physalia), contained about the same amount of copper on the fresh basis as fish, but of course these animals have a much higher moisture content.

Severy (1923) in similar studies on marine animals found shrimp to be richest in copper. Her figures for oysters were much lower than those of Rose and Bodansky and she suggests that the difference may be due to the source of the animal. Rose and Bodansky obtained their supply from the Eastern coast of the United States while Severy obtained her samples from the Western coast. When her studies were extended to two mammals which live on sea food, very little copper was found in the various tissues of these animals. No copper could be detected in the tissues of the whale, nor the blood of the sea lion. The muscles, liver, spleen, and bile of the sea lion contained traces of copper. Severy's tests like those of Rose and Bodansky were negative for clams. Severy's conclusions, "sea-lion and whale, being mammals, do not carry copper to any extent, and the copper of sea food eaten does not seem to be accumulative," are interesting because it is only very recently that the copper content of higher animals has been given serious attention.

One needs only to consult the figures for higher animals compiled by Lehmann to realize the tremendous variation in the results published up to 1900. Copper was not accepted as a definite constituent because

at the time Thudicum (1901) demonstrated that copper was present in the human brain, many workers suggested that it was only accidental and that the tissues examined might have come from people exposed to copper poisoning. However, Bodansky (1921) verified the fact that copper is a constituent of the human brain, and since that time the presence of copper in all animal tissues has been established.

Rôle of copper in marine animals. The copper-containing protein of octopus (Octopus vulgaris) blood was first obtained by Fredericq (1878). He stated that a copper compound analogous to hemin could be separated from the protein by acid and predicted that the equilibrium between oxygen and this protein was governed by factors similar to those affecting hemoglobin. Although Fredericq was the first to give to this copper compound a definite physiological function, earlier workers had suggested the importance of a compound of this nature in the blood of invertebrates. Blasius (1866) believed that the constancy with which copper was found to be present in lower forms of animal life pointed to its function in respiration. Bert (1867) considered the compound present in the blood of mollusks and Crustacea a definite respiratory pigment.

The fact that the blood of invertebrates contains hemocyanin, which is capable of forming a loose combination with oxygen, naturally interested a number of workers in the determination of the oxygen capacity of the bloods from various species. Quinquaud (1873), and Jolyet and Regnard (1877) were the first to make measurements of this nature. Their work was followed by a series of reports which have been compiled by Dhere (1919). Practically all the figures range from 1 to 5 cc. of oxygen per 100 cc. of blood except the unusually high ones of Griffiths (1892). The combined results show that the bloods of different species have unlike oxygen capacities. The respiratory capacity of the blood of mollusks, such as the octopus or the cephalopodes in general, is relatively high, three to four times that of Helix. In the case of Crustacea the respiratory capacity of the different bloods is not as high as that of the octopus, and some of the decapodes, such as Cancer and Maia, have a very low level equivalent to that of Helix.

Henze (1901) first prepared hemocyanin in crystalline form and found that, unlike hemoglobin, it could not be disintegrated into a protein and a protein-free component. According to Henze the hemocyanin of the octopus contains 0.38 per cent of copper and 1 gram of the protein combines with about 0.4 gram oxygen. This figure is one-third to one-fourth of that for hemoglobin. He used the Hopkins and Pinkus

modification of the Hofmeister ovalbumin method as a procedure for the preparation of the hemocyanin.

Quagliariello (1924) gives an excellent review of the various methods that have been used for the separation and purification of hemocyanin from blood. He also summarizes the principal properties of this protein. Dhere and Burdell (1919), and Dhere (1920) give in detail the properties of hemocyanin especially the absorption spectra of the proteins from different species. Obviously we can not go into a detailed discussion of hemocyanin here, but a consideration of the various studies on the copper content of the molecule, used to elucidate the specific differences exhibited by hemocyanins from various groups of animals and the nature of the union whereby it functions as an oxygen carrier in the blood, is timely.

Alsberg and Clark (1910) studied the elementary composition and certain of the physico-chemical characteristics of the hemocyanin from the horseshoe crab (Limulus polyphemus) and suggested that the protein of the blood of this animal differed from that of the octopus. Limulus hemocyanin was found to contain 0.28 per cent copper while octopus hemocyanin, according to Henze, contained 0.38 per cent copper. Redfield, Coolidge and Shotts (1928), who have also studied the Limulus protein, agree that it is different from that of the octopus, although their figures for the copper content are quite different from those of Alsberg and Clark. Their samples of hemocyanin prepared by different methods give in practically every case a copper content of 0.173 per cent.

The relation of the oxygen combining power to the copper content of the bloods containing hemocyanin has attracted attention since Fredericq first suggested that this compound acts as an oxygen carrier. Most of the earlier workers, Dhere (1919), felt that there was a general correspondence between the oxygen capacity and the copper content of various invertebrate bloods. Mendel and Bradley (1907) emphasized this fact when they showed that the blood of the highly active octopus contains 18 to 23.5 mgm. copper per 100 cc. while the inactive Helix pomatia contains only 6.5 to 12.5 mgm. per 100 cc. The definite relation between the copper of the molecule and its equilibrium with oxygen was questioned when Alsberg and Clark (1914) were able to obtain only 0.5 volume per cent of oxygen by subjecting Limulus blood to a vacuum after equilibration with air. This amount was so small that it could possibly be accounted for by the solubility of the oxygen in the blood itself. They suggested that the oxygen of oxyhemocyanin was not made available by diminished pressure alone, but the copper acted in

some catalytic manner in the transfer of oxygen from the blood pigment to the tissues.

Begemann (1924) was the first to study the amount of oxygen held in physical solution by the blood of invertebrates. He precipitated the proteins of the blood in his determinations, but later work by Stedman and Stedman (1925), and Redfield, Coolidge and Hurd (1926) furnishes figures for the solubility coefficient of oxygen in the unchanged blood. The method used by both authors consisted in determining the oxygen content at two oxygen pressures both of which were sufficiently great to saturate the hemocyanin completely. The latter workers found the solubility coefficient to be about 0.022 per cent for the bloods analyzed and that in nearly every case the solubility of the oxygen was lower in the blood than in sea water. These workers then demonstrated that Limulus blood has a definite oxygen capacity which is much greater than can be accounted for by solubility alone and varies from 1.66 to 0.14 volume per cent depending upon the nutritive condition of the animal from which the blood was taken. Further figures for the bloods from Loligo pealei, Busycon canaliculatum, Cancer irrocatus and Callinictus sapidus establish a definite oxygen capacity above that held in physical solution for the hemocyanin of all these bloods.

The most important question regarding the actual function of hemocyanin in respiration centers around the study of the equilibrium between oxygen, hemocyanin, and oxyhemocyanin, and the effect of carbon dioxide on this equilibrium. The work of Begemann (1924) and Redfield, Coolidge, and Hurd (1926) has shown that the same phenomena affect the gaseous exchange in bloods containing hemocyanin as in those containing hemoglobin. More recently Redfield and Goodkind (1929) point out that the reciprocal effects of oxygen and carbon dioxide upon the respiratory properties of squid hemocyanin account for one-third of the respiratory change. Parsons and Parsons (1923) studied the transport of carbon dioxide by the blood of invertebrates and concluded the hemocyanin functions in the transport of carbon dioxide in the same manner as hemoglobin. These results are also confirmed by Redfield, Coolidge and Hurd (1926) who summarize the work in the following statement:

> Studies of the conditions of equilibrium between oxygen, carbon dioxide, hemocyanin, and oxyhemocyanin show that the properties of the hemocyanin of each of these species are distinctive, but that all these proteins function in the transport of oxygen and carbon dioxide according to the same physico-chemical principles as obtained in the case of hemoglobin, although the reciprocal relation

between oxygen and carbon dioxide (the Bohr effect) is reversed in the case of certain bloods containing hemocyanin.

Since the effect of carbon dioxide is in reality due to changes in the acid base equilibrium of the blood, the actual changes depend upon the pH of the blood. The behavior of the various hemocyanins in relation to hydrogen ion concentration has been extensively investigated by Hogben and Pinhey (1926, 1927) and Stedman and Stedman (1926). According to these workers the affinity for oxygen passes through a minimum at some characteristic value of the hydrogen ion activity and that hemocyanins can be placed in certain groups depending upon their activity in this respect. The fact that an increase in carbon dioxide tension increases the affinity of Limulus hemocyanin for oxygen does not necessarily mean that this hemocyanin differs fundamentally from hemoglobin, but the actual fact observed is that the minimum is found at pH 8.0 and that if the carbon dioxide pressure is increased above this critical value the affinity for oxygen diminishes. The equilibrium of hemocyanin with oxygen is discussed in detail in an excellent review on hemocyanin by Redfield (1934).

Thus we see that the factors controlling the affinities of hemoglobin and hemocyanin for oxygen are the same and that these blood pigments are not fundamentally different because one contains iron and the other copper. The variation in the affinity of individual hemocyanins for oxygen is undoubtedly dependent upon the physico-chemical changes taking place in the protein part of the molecule.

Begemann (1924) found that the bloods of both snails and crabs unite with oxygen in a definite ratio to copper. The amount in combination suggests that one atom of oxygen unites with each atom of copper present in the hemocyanin molecule. Redfield, Coolidge and Montgomery (1928) give the combining ratio for the blood of nine species of animals which demonstrates that oxygen combines with hemocyanin in a simple stoichiometrical proportion, one atom of oxygen with one atom of copper in the molecule. It was also found that the same ratio exists in the case of the bloods of Busycon canaliculatum and Busycon carica, which, according to Mendel and Bradley (1907), contain a special hemocyanin called hemosycotypin. Therefore, the zinc present in the molecule apparently plays no part in the reaction with oxygen. The same condition is found in the case of blood from Ascidia which Henze (1911) found to contain vanadium. The hemocyanin-like compound containing vanadium was shown by Henze (1912) to be unable to hold oxygen in dissociable form.

Since all hemocyanins containing copper unite with oxygen in a definite ratio, we may conclude that there is a similar copper group common to the hemocyanins from all the different species.

Chemical work on the composition of the prosthetic group from different hemocyanins has not proved conclusively that all hemocyanins contain identical groups. It is fairly certain that at least some of the compounds prepared do not contain a porphyrin similar to that present in hematin. Conant, Dersch and Mydans (1934) suggest that the prosthetic group of Limulus hemocyanin is a complex copper salt of an unknown sulfur compound and a polypeptide, consisting of one molecule each of leucine and tyrosine and three of serine.

The copper in oxygenated hemocyanin like the iron in oxyhemoglobin remains in the reduced state. The copper in hemocyanin may be oxidized with suitable reagents to form methemocyanin. However Conant, Chow and Schoenbach (1933) have shown that methemocyanin, unlike methemoglobin, still retains its property of forming loose combinations with oxygen.

Copper in the form of turacin. Another interesting copper compound is the highly colored pigment, turacin, which is found in the feathers of the South African bird, Turaco. It was first described by Church (1869) who found it to be present in eighteen of the twenty-five known species of Mosophagidae. He found it to contain 7.0 per cent copper and suggested the following formula, $C_{82}H_{81}Cu_2N_9O_{32}$. Turacin was shown to be quite analogous to hematin and yielded, by the action of strong sulfuric acid, a colored derivative turacoporphyrin which resembled the properties of hematoporphyrin, the corresponding derivative of hematin.

Laidlaw (1904) who showed that hematin could be produced by simple union of hematoporphyrin and iron, also produced a copper porphyrin compound synthetically which resembled turacin. He found that by adding to a solution of hematoporphyrin in ammonia some cuprammonium solution and boiling, turacin was formed at once. It was purified by throwing out of solution with acid and washing with acidified water. Laidlaw believed that turacoporphyrin and hematoporphyrin were identical, because he made from turacoporphyrin and iron a hematin which agreed in all spectral characteristics with the naturally occurring pigment. The more recent work of Fischer and Hilger (1923) has shown that the turacin porphyrin is probably uroporphyrin. Uroporphyrin is occasionally excreted in the urine of man in conditions known as congenital porphyrinuria. Since the presence of free porphyrin in

the blood of animals renders them very sensitive to light, the formation of the copper compound is perhaps one means of rendering this material non-active.

Turacin gives a characteristic absorption spectrum whether examined in the feathers themselves or in a weak alkaline solution in which the pigment is soluble. The spectrum of the turacin in solution differs from that of the material when in the feathers. The reason for this difference has been explained in a very complete manner by Keilin (1926). Turacin does not combine with any of the nitrogen compounds, such as ammonia, pyridine, nicotine, or albumin, which enter so easily into combination with hematin. It is not affected by O_2 or CO in either alkaline or acid solution. Strong oxidizing or reducing agents do not change turacin. It is inactive in oxidase and peroxidase reactions although the metallic copper salts are very active. Copper when combined with porphyrin is, therefore, at the present time only associated with one type of animal, the Turaco bird. Whether further compounds of this nature are present in other animals and possibly plants and have remained unnoticed up to the present time remains for speculation.

Copper in higher animals. Investigations concerning the rôle of copper in the higher forms of animal life have been reported only in recent papers. The first definite figures presented for the copper content of organs of vertebrate animals, since the early work reviewed by Lehmann, are probably those of Bodansky (1921) who found copper in the adult brain to the extent of 3.6 to 6.0 mgm. per kgm. He analyzed one fetal brain and detected 6.8 mgm. per kgm. which led him to conclude that copper is stored more rapidly in intrauterine life, a phenomenon similar to that found for iron and iodine. McHargue (1925) made a rather extensive investigation of the occurrence of minor inorganic elements in plants and animals with especial reference to the distribution of copper. This author states, "from the wide distribution of copper in rocks, soil, plants, and sea water, its occurrence in the tissues of land and sea animals is a natural consequence but its concentration in certain organs is of unusual significance." He gives figures for the copper content of several materials of animal origin such as eggs, beef tissues, blood, rat and guinea pig organs, and fish livers. He points out the interesting fact that livers are very high in copper especially the livers of young animals. The liver from a calf shortly after birth contained 8 times as much copper as the liver from a mature ox. The chief purpose of his paper was to correlate the high copper content of materials with their high vitamin A potency. McHargue concludes

that he "is led to infer that an organic colloidal compound of copper, such as probably exists in green leaves, germs of seeds, whole milk, blood, livers, and the yolks of eggs, may be the fat soluble A factor." Of course the validity of a generalization of this nature from the few cases he presented is questionable. The mere fact that purified cod liver oil, which is an excellent source of vitamin A, is very low in copper while the residue from which the oil has been extracted is high in copper is sufficient evidence to make a statement of this sort invalid.

In a later paper McHargue (1926) presents evidence that copper, as well as manganese and zinc, is a factor in the metabolism of animals. When small amounts of these elements were added to a synthetic diet fed to rats greater growth was observed than when the rats received the synthetic diet alone. However, manganese gave better growth than either copper or zinc. The synthetic diet used was not freed from the elements in question and it contained a fair amount of all three of these elements. The copper content of the carcasses of the rats receiving the copper was slightly higher than that of the control animals.

The copper content of milk has also received considerable attention. The figures given by a few of the earlier investigators, McHargue (1925), Guerithault (1927), Fleurent and Levi (1920) vary from 0.38 to 1.4 mgm. of copper per liter. Supplee and Bellis (1922) found the average of 23 samples of cow's milk to be 0.52 mgm. per liter. In a later paper Hess, Supplee and Bellis (1923) reported that the copper content of commercially pasteurized milk was from 0.6 to 0.7 mgm. per kgm. The milk from women on a mixed diet was found to contain 0.4 to 0.6 mgm. per liter. They therefore concluded that infants were continually ingesting copper whether fed human milk or cow's milk. Their studies were extended and showed that a three months old infant observed for seven days excreted in the urine 0.02 mgm. copper daily. Older infants, two to three years of age, excreted from 0.06 to 0.14 mgm. copper per liter of urine. An adult on an ordinary diet excreted 0.08 to 0.09 mgm. copper per liter, and another adult on a high copper diet eliminated 0.11 to 0.14 mgm. copper per liter. In conclusion these authors state, "Whatever may prove to be its physiological or pathological significance, our investigations show that cow's milk and woman's milk regularly contain copper, and furthermore, that an infant's and adult's copper is absorbed from the alimentary tract as proved from its constant presence in the urine." Workers were not only interested in the copper content of milk because of its physiological importance but also because the presence of copper brings about certain typical changes in the milk itself, especially

off-flavors. Hess and Unger (1921–22) have shown that if milk is contaminated with copper the antiscorbutic potency of the milk is greatly reduced. Both of these changes indicate that copper may play the rôle of catalyst in certain organic reactions.

The more recent work of Elvehjem, Steenbock and Hart (1929a) sets the figure for the copper content of milk at a somewhat lower value. Milk produced by cows on a normal ration was found to contain 0.15 mgm. copper per liter. Samples of cow's milk collected from herds in different sections of the U. S. and fed different rations showed very slight differences in copper content. The copper content of the milk was not increased by feeding the cows sufficient copper sulfate to increase the copper intake five-fold. Similar results were obtained when the milk from goats fed increased amounts of copper was studied. The copper content of goat's milk was not found to be appreciably lower than that of the cow as Quam and Hellwig (1928) reported. These workers also analyzed milk that has passed through various manufacturing processes. Pasteurized whole milk contained more copper than raw milk, and buttermilk contained a considerable amount. Condensed and evaporated milks both contained more copper than could be traced to the milk in its original state.

Copper in experimental anemia. The association of copper with a definite function in the animal body was shown by work at the University of Wisconsin. This work was initiated in 1924 and consisted of studies in nutritional anemia and factors affecting hemoglobin regeneration in this disease. Rabbits were used in the original work (Hart, Steenbock, Elvehjem and Waddell, 1925), and when these animals were placed on a diet of whole milk at weaning time an anemia, characterized by a low hemoglobin content and erythrocyte count of the blood, developed. Inorganic iron, in the form of ferric oxide, added to the basal diet did not correct this condition unless accompanied by fresh cabbage or an alcoholic extract of cabbage or yellow corn. These extracts were free from iron. Later work by Hart, Elvehjem, Waddell and Herrin (1927) showed that the ash of lettuce or cabbage added together with ferric oxide to the whole milk diet greatly stimulated hemoglobin formation. The authors conclude, "It is apparent that a deficiency in milk leading to nutritional anemia is of inorganic character rather than of organic nature." Impure soluble iron salts were found more effective than the more purified preparations, again suggesting the importance of inorganic factors.

In the more recent work (Hart, Steenbock, Waddell and Elvehjem,

1928) where rats were used as the experimental animals, the ineffectiveness of highly purified iron salts in the restoration of hemoglobin in the blood of anemic rats was definitely demonstrated. The ash of liver, lettuce, and corn, was also found very effective in the cure or prevention of anemia in this animal. The liver extract described by Cohn and associates (1927) found to be very effective in the treatment of pernicious anemia in humans was also tried. The ash of this material was found very potent when fed together with a soluble iron salt. The active element was precipitated by hydrogen sulfide from an acid solution of the ash. The pale blue color of the ash of this material suggested that copper was possibly the active element. The addition of 0.25 mgm. of copper as copper sulfate and 0.5 mgm. iron as ferric chloride to the whole milk diet of an anemic rat produced an immediate and striking recovery. The hemoglobin in the blood increased from 2.7 grams per 100 cc. to 13.3 grams in a period of six weeks. As the authors suggest, this is undoubtedly the first experiment recorded in the literature giving to copper in association with iron the specific function of hemoglobin formation in a mammal on an otherwise satisfactory diet. The feeding of different levels of copper showed that 0.05 mgm. of copper together with 0.5 mgm. iron gave optimum responses. Liver extract was found to contain 0.016 per cent of copper, each daily feeding of this material therefore supplied approximately 0.05 mgm. of copper. It was very low in iron and therefore ineffective unless fed together with an iron salt.

The controversy which existed in the literature for the past half century over the effectiveness or ineffectiveness of iron salts in the cure and prevention of anemia cannot be reviewed here but undoubtedly the question depends upon the amount of copper supplied along with the iron. Waddell, Steenbock and Hart (1929) point out that carefully purified iron salts can be fed at high levels to anemic rats without showing any tendency to correct the anemia. Elvehjem and Lindow (1929) found an average of 0.02 mgm. copper per gram of iron salt in the majority of the salts tested.

McHargue, Healy and Hill (1929) confirmed the observations of the Wisconsin workers when they showed that anemic rats fed the ash of calves' liver maintained distinctly higher hemoglobin values than a similar group of rats fed the ash of the liver from which the copper had been removed. Krauss (1929) also demonstrated that anemia produced in rats fed an exclusive milk diet was not cured by the addition of iron salts alone, but when iron salts were supplemented with copper salts the blood picture rapidly returned to normal. Titus, Cave and Hughes

(1929) using similar experimental conditions also found that copper together with iron salts was very effective in the cure of nutritional anemia. These workers, however, consider that manganese added to the milk and iron diet is almost, though not quite, as efficient as the addition of copper for hemoglobin building. A careful study of their curves shows that there is a considerable lag in the rate of hemoglobin regeneration when manganese is fed in place of copper; however, the figures are definitely higher than the controls. Experiments have been repeated in the Wisconsin laboratory, Waddell, Steenbock and Hart (1929a), using the same manganese salt which Titus used and entirely negative results were obtained. Not only did they find that manganese was inactive but that a combination of twelve elements consisting of zinc, nickel, chromium, germanium, cobalt, lead, antimony, tin, cadmium, mercury, arsenic and manganese was inactive. Whether these different results were due to a variation in the composition of milk used in the two laboratories or to other factors is difficult to say.

Several other workers presented evidence to show that iron alone is effective in curing nutritional anemia in rats or that copper is non-specific as a supplement to iron. Mitchell and Vaughn (1927) grouped the iron compounds into good, fair, and poor sources of iron. Salts such as ferric acetate, ferric albuminate, ferric chloride, and ferric citrate gave excellent recoveries when fed alone. Myers and Beard (1929) found that traces of manganese, nickel, copper, germanium and arsenic when fed with iron all had a definite supplementing action on hemoglobin regeneration. They suggested their results with iron alone were in agreement with those of Mitchell and Vaughn. At about the same time Mitchell and Miller (1929–30) found spinach extract to be more potent than a solution of iron, copper, and manganese containing the same quantities as found in the spinach extract. They concluded that there is a group of elements active in hemoglobin building. Beard and Myers (1931) concluded that inorganic iron prevents and cures nutritional anemia, although traces of many other elements do have a supplementing action on small amounts of iron, and that the specificity of copper as a supplement to iron receives little support from their work. Keil and Nelson (1931) also reported that iron can be utilized directly but the action of it is catalyzed by copper. Drabkin and Waggoner (1930) showed that a nutritional anemia induced in rats by a whole milk diet could be cured by transferring the rats to a synthetic diet containing iron, but which they claimed to be copper free. Later Drabkin and Miller (1931) concluded that certain amino acids, especially arginine

and glutamic acid, when added to milk supplemented with iron, were very effective for the relief of anemia.

It should be noted that all of these statements have been retracted or refuted in more recent papers. In a later paper Mitchell and Miller (1931) attribute the slow increase in the hemoglobin of anemic rats fed iron alone to traces of copper available from the milk supply or stored in the animal body. Keil and Nelson (1931a) have also suggested that the recoveries with iron alone which they observed in their first work were due to copper present in the milk. The copper content of the milk used in those experiments was 0.35 to 0.44 mgm. per liter, while that used in later experiments when iron alone was ineffective was 0.24 mgm. Lewis, Weichselbaum and McGhee (1930), Krauss (1931), Orent and McCollum (1931), Keil and Nelson (1932, 1934b), Orten, Underhill and Lewis (1932) have all studied the value of manganese as well as a number of the other elements reported to be active by Beard and Myers and have found all to be inactive except copper. The animals used by Beard and Myers must have ingested from the food or surroundings or metabolized from stores in the body sufficient copper to bring about hemoglobin regeneration when iron was fed alone. In a recent report from Myers' laboratory (Myers, Remp and Bing, 1934) a statement is made that no improvement in the hemoglobin content of the blood of anemic rats on carefully controlled milk was obtained with iron alone.

The results of Drabkin may be explained by the presence of copper in the synthetic diet or amino acids used. Elvehjem and Hart (1931) found the synthetic diet used by Drabkin to contain appreciable amounts of copper. The ineffectiveness of pure glutamic acid as a supplement to iron has been demonstrated by Elvehjem, Steenbock and Hart (1931) and Keil and Nelson (1932). Rider (1933) reported that glutamic acid renders iron and copper therapy more effective. Brand and Stucky (1934) suggest that glutamic acid may have a hematopoietic effect in young rats from mothers on a low protein diet. In experiments with young rats from their stock colony they (Brand and Stucky, 1934a) found that the addition of glutamic acid to various metals did not produce any supplementary effect on hemoglobin regeneration. Fitz-Hugh, Robson and Drabkin (1933) found both iron-copper and iron-sodium glutamate effective therapeutic agents in nutritional anemia in rats, and that the latter was somewhat superior. The diet of the mother may have some effect upon hemoglobin building in young, but there can be no question but that milk is deficient in only iron and copper for hemoglobin building in rats reared by mothers on ordinary rations be-

cause hundreds of rats have been cured of their anemia and maintained with a normal blood stream for considerable periods on milk plus iron and copper. Underhill, Orten, Mugrage and Lewis (1933) found a normal blood picture in rats continued on a milk, iron and copper ration for 667 days.

Kemmerer, Elvehjem and Hart (1931) working with mice, and Skinner, Peterson and Steenbock (1932) working with rats, have demonstrated that the addition of traces of manganese to a diet of whole cow's milk supplemented with iron and copper has a favorable effect upon growth. Later work (Kemmerer, Elvehjem, Hart and Fargo, 1932) has shown that rats on cow's milk mineralized with iron, copper and manganese made average daily gains very similar to those made by rats on an ordinary ration. The question of reproduction on this diet cannot be discussed in this review, but much remains to be done on this problem. Studies by Elvehjem, Hart, Jackson and Weckel (1934) indicate that growth and reproduction depend to a large extent upon seasonal variations in milks used.

It might be well to state very briefly some of the precautions which must be taken to insure accurate results in nutritional anemia studies. The rats should come from mothers of known history and the ingestion of food high in iron or copper during the suckling period should be reduced to a minimum. The animals should be caged in such a way as to prevent contact with iron and copper. Most workers have found galvanized iron wire cages very good. The relative value of wire and glass cages has been studied by Skinner, Steenbock and Peterson (1932) and Geraghty, Underhill, Orten and Lewis (1933). The milk should be handled carefully as has been pointed out by Keil and Nelson (1931). The fact that milk is collected directly into glass vessels is no guarantee against copper contamination unless the glass utensils are very carefully washed. The use of a standard milk powder has been recommended by Harris (1932) and Remington (1933). The anemia should be developed rapidly so that no degenerative changes take place. The method outlined by Elvehjem and Kemmerer (1931) has proved very satisfactory. The hemoglobin content of the blood of the rats should be below 3 grams per 100 cc. before the animals are started on experiment. If these precautions are taken it is very simple to demonstrate experimentally that copper is necessary as a supplement to iron for hemoglobin formation, that of a large group of elements copper is unique in this respect, and that milk is deficient in only iron and copper as far as hemoglobin formation is concerned.

These conclusions need not be limited to rats because the activity of copper has been demonstrated in chickens by Elvehjem and Hart (1929a), in pigs by Elvehjem and Hart (1932), in lambs by Thomas and Wheeler (1932).

The value of copper as a supplement to iron for hemoglobin formation in dogs has been studied only in the prolonged anemia type of experiment used by Whipple, Robscheit-Robbins and co-workers (1925–27). It should be kept in mind that in these experiments no attempt was made to prevent copper ingestion or reduce copper storage. Dogs are placed on a standard bread diet which keeps the hemoglobin production at a low level. During the first period of two to three months hemoglobin is removed by bleeding to compensate for the reserve storage of certain hemoglobin building factors. After this period the amount of hemoglobin in the blood of the dog remains at 40 to 50 per cent of the normal and the amount produced over and above this level is generally not more than 1 to 3 grams per week. The hemoglobin building power of different food materials can thus be studied by determining the amount of hemoglobin produced weekly when the various substances supplement the basal diet. These workers observed very early in their studies that apricot feeding had a very beneficial effect on this type of anemia in dogs. Later they found that the ash of dried apricots retained most of the potency of the original fruit.

The ash of beef liver and pig kidney was likewise found to be potent although some of the activity was lost during the ashing process. In the paper by Robscheit-Robbins, Elden, Sperry and Whipple (1928) which included the above results, the authors state, "Our results are not wholly in accord with the observations of the Wisconsin workers, but the divergent results are to be explained by differences in the anemia or in the experimental animal." There is evidently no difference in the mechanism of hemoglobin formation in dogs and other experimental animals but some methods of anemia production may emphasize certain deficiency factors over others. In a later paper by Elden, Sperry, Robscheit-Robbins and Whipple (1928) when iron and copper salts were fed directly to the anemic dogs, they conclude "combined copper and iron feeding may at times have an unusually favorable effect or again may not exceed the favorable influence of iron salt feeding alone"; and "The influences of copper tartrate or sulfate added to the standard diet in our experiment is slight and much less than iron salts or the ash of apricots, liver or kidney." Practically negative results are to be expected when copper is fed alone because there is then insufficient iron present to act

as a building stone for the hemoglobin molecule. When the ash of liver, kidney or apricots is fed, sufficient amounts of both elements are supplied, but when iron salts are fed alone good or fair results are obtained depending upon the amount of copper supplied in the basal diet or available from the stores in the body. It should be kept in mind that when the dogs are bled to remove hemoglobin large amounts of iron are withdrawn but only traces of copper. Therefore the animal will be depleted of iron before copper. This method also allows the removal of organic constituents of the blood and may prove very useful as Whipple (1934) has suggested for the study of the precursors of organic constituents of the blood.

We are probably safe in concluding from the experimental work reviewed that copper is of fundamental importance in the formation of hemoglobin in all red-blooded animals. This does not mean that some of the other elements such as manganese, zinc, etc., which have been mentioned are not essential in the living organism, but it does mean that they are not directly concerned in hemoglobin formation.

Copper in foods. The establishment of copper as an essential element in nutrition makes the question of the supply of this element in normal foodstuffs an important one. Early work concerning the detection of copper and analyses of certain plant and animal tissues has already been reviewed but no comprehensive study of a large number of foods is given in these papers. The analysis of a large number of different samples was made possible only when methods which required relatively small samples and which were not too long or tedious became available. The early methods consisted of precipitation of the copper with H_2S, converting the precipitate to CuO and weighing as such. Later methods made use of the blue color formed by the addition of ammonia or the red color with potassium ferrocyanide. The electrolytic method of Pregl (1924) was also used by some workers.

The methods in general use today are dependent upon color reactions. The potassium ethyl xanthate method was adapted to milk by Supplee and Bellis (1922) and the adaptation was used by King and Etzel (1927) and by Quam and Hellwig (1928). The Biazzo (1926) method, which is dependent upon the Spacu (1924) reaction was developed for general use by Elvehjem and Lindow (1929) and by Schönheimer and Oshima (1929). Slight modifications in this method have been made by Gebhardt and Sommer (1931), Kleinmann and Klinke (1930), Drabkin and Waggoner (1930), and Elvehjem and Hart (1931). Callan and Henderson (1929) used diethyl dithiocarbamate for the development of the

color with copper and extracted the colored compound with amyl alcohol. This method has been used by McFarlane (1932) and Locke, Main and Rosbash (1932). A titrimetric method involving nitroso-chromotropic acid has been used by Cherbuliez and Ansbacher (1930) and Ansbacher, Remington and Culp (1931). Fischer and Leopoldi (1934) have used diphenylthiocarbazon and extract the color from acid solution with carbon tetrachloride. Four gamma of copper are readily determined by this method. Sarata (1933) has developed a method which depends upon the fact that a dilute solution of m-benzaminosemicabazid gives a red color with copper and which can be used for amounts of copper as low as 0.01 gamma. Roncato and Bassani (1934) have used a polarographic method.

Warburg (1927) has determined copper by measuring the rate of oxidation of cysteine in properly buffered solutions manometrically. Dutoit and Zbinden (1930), Fox and Ramage (1931), and Sheldon and Ramage (1931) have used spectrographic methods.

The most comprehensive figures for the copper content of food materials are given in the papers by Guerithault (1927), Quartaroli (1929) and Lindow, Elvehjem and Peterson (1929). The results given in the three different papers agree very well when one considers that the work was done in different countries. Of the 158 samples analyzed by Lindow, Elvehjem and Peterson, no food was found to be completely lacking in copper. The figures range from 0.1 mgm. of copper per kgm. of fresh celery to 44.1 mgm. per kgm. of fresh calf liver. The foods were classified in descending order of copper content as follows: nuts, dried legumes, cereals, dried fruits, poultry, fish, animal tissues, green legumes, roots and tubers, leafy vegetables, fresh fruits and non-leafy vegetables. The degree of variation in the copper content of foods in the same class was less than that found for iron and manganese. Several other workers (Webster and Jansma, 1929; Remington and Shiver, 1930; Cunningham, 1931; Schuette and Remy, 1932) have studied the copper content of certain groups of foodstuffs. Davies (1931) has studied the copper content of milk and dairy products such as butter and cheese, and Davies (1933) the distribution of copper between the constituents of milk. Kondo and Mori (1932) also studied the distribution of copper in milk. Zondek and Bandmann (1931) found 3 to 4 times as much copper in human milk as cow's milk, but Remy (1932) found very little difference. A very recent paper by Conn, Johnson, Trebler and Karpenko (1935) includes a review of much of the work on the copper content of milk. These workers found the copper content of uncontaminated raw milk to range from 0.051 to 0.132 p.p.m.

Elvehjem, Kemmerer, Hart and Halpin (1929) and Cunningham (1931) have studied the copper content of eggs and found that the amount of copper in eggs was not influenced by feeding the hens extra copper. Erikson, Boyden, Martin and Insko (1933) found that hens allowed sunshine, bluegrass range, and cod liver oil produced eggs with a higher copper content in the yolk than those from hens on a basal ration. Elvehjem and Hart (1929) were able to increase the copper content of lettuce 148 per cent by growing it on soil fertilized with $CuSO_4$ equivalent to 500 pounds per acre. Miller and Mitchell (1931) observed similar effects in spinach and lettuce grown on soil treated with copper sulfate.

Hodges and Peterson (1931) have calculated the daily copper intake for sixteen menus given by Rose and found it to vary from 0.8 mgm. for a child three to four years old to 4.81 mgm. for the working man. That the amount of copper in an ordinary diet does not greatly exceed the requirement, especially in children, is readily evident from the recent studies by Daniels and Wright (1934). They suggest that the diet for children of pre-school age should include not less than 0.1 mgm. of copper per kilo of body weight. Many diets may not supply this amount of copper. It should also be pointed out that all of the copper in food may not be available. Schultze, Elvehjem and Hart (1934) found the copper of copper caseinate, glycine amide biuret, alanine amide biuret, hemocyanin and whole wheat to be readily utilized by severely anemic rats, but copper hematoporphyrin was not utilized even when fed at high levels. How much of the copper in natural foods, if any, occurs as copper porphyrin is not known. Sherman, Elvehjem and Hart (1934) have demonstrated that egg yolk inhibits the utilization of copper when the copper is present in small amounts.

The copper content of feeds used for livestock has not been given much attention. Elvehjem and Hart (1929) analyzed 47 common feeds and found the average copper content to be about one-fifth the manganese and one-fifteenth of the iron content. The most interesting studies are those that have been made on the pasture grasses from areas where cattle are known to be suffering from anemia. Anemia in grazing animals has been reviewed by Davidson and Leitch (1934). The best known areas which have been shown to be deficient in copper are those reported by Neal and Becker (1933) in Florida and Sjollema (1933) in the Netherlands.

Copper in the treatment of anemia. Since copper is necessary as a supplement to iron for hemoglobin formation serious consideration

should be given to the relation of this element to the treatment of anemia. It should be kept in mind that copper supplies only one deficiency and is of no value unless accompanied by iron or other factors necessary for normal blood formation. Early medical literature contains several references to the use of copper in anemias (reviewed by Liegeois, 1901) but no attempt was made to use a combination of available iron and copper until the experimental work already reviewed was reported.

The demonstration that inorganic iron can serve as the sole source of iron for hemoglobin formation reëmphasized the value of simple iron salts in the treatment of anemias. There has been a tremendous increase in the clinical use of salts such as ferric ammonium citrate, ferric sulfate, etc., during the last few years. The insurance of proper utilization of iron in the presence of adequate copper allowed a more complete knowledge of the availability of different forms of iron. Elvehjem (1932) showed that hematin was a much poorer source of iron than simple salts like ferric chloride. Additional studies by Elvehjem, Hart, and Sherman (1933, 1934) have shown that the availability of iron in foods may vary from 25 to 80 per cent of the total iron content. Thus it is impossible to evaluate foods as a source of iron in the diet upon their total iron content.

What portion of the cases suffering from hypochromic anemia need both iron and copper and what portion will respond to iron alone cannot be answered at the present time if ever. However, a number of workers (Lewis, 1931; Josephs, 1931; Bloxsom, 1932; Caldwell and Dennett, 1932; and Cason, 1934) have presented evidence to show that iron and copper given in combination are more effective in the treatment of nutritional anemia in children than iron given alone. These results are not surprising since it has already been mentioned that the copper intake on ordinary infant dietaries does not greatly exceed the requirement. The fact that Ramage, Sheldon and Sheldon (1933) found a reduced copper content of the livers of infants during the nursing period and an increase as soon as a mixed diet was supplied also indicates the possible need of copper. Parsons and Hawksley (1933) and Mackay (1933) are inclined to believe that copper deficiency does not play any important part in common nutritional anemia of babies in England. However, Parsons and Hawksley suggest that in some cases the cure of nutritional anemia will not take place unless an adequate amount of copper is given and Mackay points out that her negative results do not show that copper deficiency never occurs in babies suffering from iron deficiency. Görter,

Grendel and Weyers (1931) conclude from a study of the copper content of the blood of infants that there is no shortage of copper during nutritional anemia.

Dwyer (1930), Mills (1930, 1931), Waugh (1931), Adamson and Smith (1931), Gross (1932), Dameshek (1933), Wintrobe and Beebe (1933) and Machold (1934) have observed better results in certain types of anemia in adults by the use of iron plus copper than with iron alone. Heath (1933), Bethell, Goldhamer, Isaacs and Sturgis (1934), and Davidson and Leitch (1934) conclude that in the treatment of the usual cases of iron-deficiency anemia no advantage is gained by including copper in therapeutic preparations of iron. However Davidson (Fullerton, 1934) has suggested the use of tablets containing ferrous sulfate and copper sulfate in the ratio of 100:1. It is impossible to attempt to discuss in detail the data presented in the above papers and attempt to explain the divergent results. The difficulties encountered in obtaining clinical data of this nature are, of course, very great. The most reasonable conclusion seems to be that available iron accompanied by small but standard amounts of copper should be used in all cases of anemia showing reduced ability to form hemoglobin. Sheldon (1932) states that there is sufficient evidence to justify the giving of small amounts of copper as a supplement to iron used in the treatment of anemia.

It is generally accepted that iron and copper are ineffective in pernicious anemia. Middleton (1928) feeding an iron and copper solution in two cases of pernicious anemia observed no improvement after fifteen days of treatment. He did note, however, a very sharp rise in the reticulocytes within a few hours after the initiation of the liver extract therapy. The use of iron and copper together with liver extract, especially in the case of highly purified extract, may be of value in aiding hemoglobin building.

Many workers have found iron and copper salts very effective in combating nutritional anemia in suckling pigs (Hart, Elvehjem, Steenbock, Bohstedt and Fargo, 1929; Hamilton, Hunt and Carroll, 1933). Several papers have appeared on the use of copper in the treatment of diseases such as tuberculosis, leprosy, etc., and on the protective action of copper against certain infections, but more work is needed before definite conclusions can be drawn.

Rôle of copper in plants and microörganisms. Hoagland (1932) has reviewed the recent work concerning the value of copper for the growth of higher plants. Sommer (1931) and Lipman and Mackinney (1931) have produced definite evidence that copper is an essential element for

plant growth. Working with purified water cultures, these investigators found that plants such as barley, flax, tomatoes, and sunflowers showed a much greater vegetative growth when traces of copper were added. Lipman and Mackinney showed that barley plants were unable to produce seed without the presence of copper in the culture medium. From a practical point of view Felix (1927) and Allison, Bryan and Hunter (1927) have improved the production of a large variety of plants on peat soils by the addition of copper. Brenchley (1932) found no improvement by the addition of copper to peat soils in England. Bryan (1929) has produced greening in chlorotic leaves grown on peat soils by treatment with solutions of copper sulfate or manganese sulfate. Orth, Wickwire and Burge (1934) studied the effect of copper on the chlorophyll content of "frenched" orange tree leaves and found the leaves from trees treated with copper to contain 4.6 times as much chlorophyll as the untreated leaves.

Little work has been done on the relation of copper to the growth of microörganisms because it is very difficult to remove the last traces of the element from the cultures used. Bortels (1927) investigating the importance of iron, zinc and copper for the growth of Aspergillus niger found that copper stimulated the formation of the black conidial pigment of the mold. He also concluded that copper had no effect on the growth of yeast. Wolff and Emmerie (1930) found copper to be necessary for both growth and sporulation of Aspergillus niger. Elvehjem (1931) prepared a synthetic culture medium which was exceedingly low in iron and copper. Bakers' yeast made a meager growth on this medium and the yeast produced was characterized by a pale color, low iron content, complete absence of free inorganic iron, low cytochrome content, and low respiratory quotient. Parsons and Hickmans (1933) obtained a similar yeast on purified synthetic media. The addition of iron alone accelerated the rate of growth and increased the cytochrome content and rate of respiration. The addition of both iron and copper gave a further increase in rate of growth and caused the production of cytochrome with a distinctly higher *a* component. Thus copper affects the formation of hematin compounds in other living matter besides the animal organism. The addition of 0.02 mgm. of copper per 200 cc. of medium was found to be the optimum amount for stimulating the formation of cytochrome *a*. Larger amounts of copper inhibited cell growth during the first 48 hours but the toxic effect disappeared as the number of cells per unit of medium increased.

McHargue and Calfee (1931) studying the growth of yeast in a puri-

fied medium found that small quantities of manganese, copper and zinc increased the dry weight of yeast produced. Larger amounts of these elements were toxic in all cases. Copper stimulated cell division and was also more effective than manganese or zinc in stimulating carbon dioxide production.

The mechanism of copper activity. Very little is known about the mechanism by which copper acts in biological processes. In the plant kingdom we can merely say that copper seems to be associated with the most active parts of the plant, but the type of compound present is entirely unknown. In the animal organism studies have centered around the distribution of copper in the tissues, changes in the copper content of blood, the effect of copper upon iron storage and utilization, and its relation to certain enzyme systems.

Lindow, Peterson and Steenbock (1929) made detailed studies on the assimilation, storage, transmission to the offspring, and excretion of copper in the rat. They found the absolute amount of copper in a rat to be 0.0108 mgm. at birth and that it increased continually with growth to a value of 0.4422 mgm. at about 200 days of age. The daily storage was about 0.001 mgm. for the first 12 days and about 0.002 mgm. thereafter. The increased storage after 12 days was undoubtedly due to the consumption of the grain ration in addition to the mother's milk. While the absolute amount of copper per animal increased with age, the percentage decreased during the first 12 days but remained fairly constant during the next 200 days. This indicates that the amount of copper follows rather closely the total mass of active cells. When rats were fed the normal ration plus 5 mgm. copper daily the young at birth contained no more copper than young from mothers on the normal diet. Cunningham (1931) however found a small difference between the copper content of young from mothers on a low copper diet and those from mothers receiving 7.5 mgm. copper daily. Hart et al. (1930) found the livers from pigs farrowed by sows receiving added copper to contain slightly more copper than livers from pigs farrowed by sows on the basal ration. More data are needed on the effect of the diet of the mother on the copper content of the young.

Feeding copper directly to the young rats increased the copper content of the entire body 2 to 3 times. If the young were placed on a milk diet the amount of copper in the rat was the same at 3 weeks of age and at the time of death due to anemia (9–11 weeks). The addition of 1 mgm. of copper daily per animal increased the copper from 0.0521 mgm. to 0.1890 mgm. Bing, Saurwein and Myers (1934) found the

copper content of their anemic rats to be slightly higher, 0.066 to 0.077, and increased the copper content to about 0.1 mgm. by feeding 0.025 mgm. Cu daily for 17 days. The daily addition of 5 mgm. of copper to a normal ration of rats increased the copper content of the skeleton, the kidney, the spleen, and the liver, 1.6, 2, 5 and 20 times, respectively, over the normal amount. Flinn and Inouye (1929) found a similar increase in the liver of rats which received 2 mgm. of copper as $CuCl_2$ daily in their drinking water for a period of 12 months. Cunningham also found a very definite increase in the liver due to copper feeding although his figures were somewhat lower. Coulson, Remington and Lynch (1934) suggest that the amount of copper assimilated depends upon other constituents of the basal ration.

Lindow, Peterson and Steenbock studying the excretion of copper found rats on a normal diet excreted 2 parts of copper in the feces to 1 part in the urine. When the stock diet was supplemented with copper, about 98 per cent was excreted in the feces and the amount in the urine increased about 5 times. The copper stored in the body was eliminated during the 4 to 5 weeks of post-period feeding. Cunningham found that iron feeding had no effect on copper excretion. Rabinowitch (1933) found the copper content of human urine to range between traces to 0.7 mgm. per 24 hour period. In two subjects fed copper the amount was appreciably larger. Sandberg and co-workers (1934) report that copper excretion is increased after splenectomy.

In addition to the relatively large amount of copper in the liver and the changes due to diet, the variations with age indicate that copper metabolism is closely associated with this organ. McHargue (1925) was the first worker to show that the livers from young animals contained more copper than livers from adults. Morrison and Nash (1930) reviewed the studies on the copper content of livers from humans of different ages and gave figures to show that infants' livers contain about 24 mgm. per kilo fresh tissue while adult livers contain only 4 mgm. Cunningham reports a similar relationship for a number of different animals. In practically all cases the percentage of copper was higher in the livers of the newly born or fetal livers than in those of adult animals. Ramage, Sheldon and Sheldon (1933) found for the human liver that the copper concentration was greater at birth than at any time during intrauterine life. Wilkerson (1934) working with pig embryos has shown that there is a continual increase in amount of copper per embryo throughout the embryonic period but the percentage of copper in the fresh tissues increases only up to the 30 mm. length stage

and decreases thereafter. The percentage of copper in the fresh embryonic livers remains fairly constant while the embryo grows from 10 mm. to 160 mm. in length.

Loeschke (1931) found the copper content of the egg to be concentrated in the liver of the chick embryo during hatching. McFarlane and Milne (1934) have studied the daily changes in the copper content of the livers of chick embryos from the 10th day of incubation to hatching, and found the absolute amount of copper in the liver to increase up to the 17th day after which it remains fairly constant. The percentage of copper in the fresh liver remains quite constant throughout the embryonic period while the copper per unit of dry liver decreases after the 14th day. McFarlane and Milne explain the similar results for the pig and chick and the divergent results for the human upon the fact that the pig and chick embryo may need copper at an earlier period because the hemoglobin is built from simpler compounds in these animals. Undoubtedly other factors also enter into these differences.

The percentage of copper in adult livers from different species is fairly constant; however, the amount of copper in beef liver seems to be unusually high and is accompanied by a low iron content. This is readily evident from the following figures which I have compiled from published and unpublished values in our laboratory and from certain values in the literature.

	Percent Fe	Percent Cu	Fe:Cu
Pig	0.083	0.0021	40.0
Beef	0.024	0.007	3.4
Rat	0.042	0.0012	35.0
Human	0.042	0.0024	17.5
Chicken	0.050	0.0018	27.7
Horse	0.090	0.0021	42.8

Although these figures are subject to considerable variation, there is no question but that the Fe:Cu ratio for beef liver is much less than for any of the other animals. It is impossible at present to suggest that the difference is of any significance. Meyer and Eggert (1932) have observed the same relationships. They also found that only part of the copper in livers was extracted with water and that a large share of the soluble copper followed the fraction precipitated with alcohol.

The copper content of blood has been studied with considerable interest. Lehman (1895), McHargue (1925) and Warburg and Krebs (1927) reported small but definite amounts of copper in whole blood and in serum before the relation of copper to hemoglobin formation was known.

Abderhalden and Möller (1928) reported 0.19 mgm. copper per 100 cc. of horse serum. Schönheimer and Oshima (1929) found human blood to contain 0.113 to 0.144 mgm. per 100 cc. Elvehjem, Steenbock and Hart (1929) found the corpuscles in horse blood to contain more copper than the serum. They also found traces of copper in crystalline hemoglobin but concluded that it could not be a constituent of the hemoglobin molecule. Guillemet and Gosselin (1932) suggest that the copper found in hemoglobin is contained in the crystal water. More recently the copper content of blood has been studied by Guillemet (1931, 1932), Grendel (1930), Herkel (1930), Kosaka (1931), Schindel (1931), McFarlane (1932), Sarata (1933a), Tompsett (1934) and Roncato and Bassani (1934). Tables showing values obtained by different workers are given by Sarata and Tompsett. Most of the values for whole blood from mammals fall between 0.05 and 0.25 mgm. per 100 cc.

There is still some question concerning the distribution of the copper between the blood constituents. Guillemet found more copper in the plasma than in the whole blood of the ox, pig and dog; Schindel found a similar distribution in human blood; but Tompsett found an equal distribution between plasma and corpuscles in the blood of several different animals. However, the values for the plasma reported by Schindel and Tompsett are very high (about 0.2 mgm. per 100 cc.) while those of Guillemet are lower and more in line with those reported by Locke, Main and Rosbash (1932) and Krebs (1928) for human serum (about 0.08 mgm. per 100 cc.). Sarata (1933b) studying the distribution of the copper between plasma and serum of man, ox, horse and rabbit has verified the original findings of Elvehjem, Steenbock and Hart (1929) that the major part of the total blood copper is present in the corpuscle fraction. Suzuki and Sarata (1933) found erythrocytes to contain far more copper than the leucocytes from horse blood. Flinn and Inouye (1929) found 1 to 2 mgm. Cu per 100 grams plasma and about 1 mgm. Cu per 100 gram corpuscles in the blood of cats which had received an injection of 10 cc. saline solution containing 100 mgm. copper into the intestine 45 to 60 minutes before bleeding.

Some attempt has been made to correlate changes in the blood copper with anemic conditions. Warburg and Krebs (1927) found an increase of 300 to 600 per cent in the serum copper following hemorrhage in the pigeon and goose. Guillemet (1932a) and Locke, Main and Rosbash (1932) found a reduction of blood copper due to repeated bleedings in dogs and rabbits. Sarata and Suzuki (1933) however found a marked rise in blood copper after hemorrhage. The high level of copper lasted

only a day or two and dropped rapidly at first and then more slowly until the normal level was reached. The changes in copper followed the reticulocyte response rather closely although the peak of the copper response preceded slightly the maximum of reticulated cells. They found the percentage increase in copper was decidedly greater in the red cells than in the plasma, indicating that the cells poured into the circulation during repair contain more copper. Beard, Baker and Myers (1931) studying the effect of iron and copper on the reticulocyte response in anemic rats concluded that iron alone aids in the production of new reticulocytes and that copper speeds up the maturation of the red blood cells. Schultze (1932) and Stein and Lewis (1933) have also reported a specific effect of copper on red cell production. Schultze and Elvehjem (1933) have studied this question using carefully controlled animals and conclude that neither iron alone nor copper alone can produce a typical reticulocyte response in rats suffering from severe nutritional anemia. When adequate amounts of both iron and copper were fed a typical response occurred. The intraperitoneal injection of blood hemoglobin into anemic rats caused a reticulocyte response similar to that obtained with suboptimal amounts of iron and copper. This fact indicates that cell formation is not dependent directly upon either iron or copper but apparently stimulated by products formed upon the interaction of both the metals in the body. The relationship of copper to these changes is very difficult to study because such minute quantities of copper are required to produce definite changes. The improved methods for determining exceedingly small amounts of copper which have been developed will aid in determining the exact relation of blood copper changes to cell formation.

Locke, Main and Rosbash (1932) have made further observations on the changes in blood copper and non-hemoglobinous iron and the ratio of these elements in the blood during various diseases. However, more data are necessary before the generalizations which they suggest can be made.

The effect of copper upon iron storage and utilization has been studied by Cunningham (1931), Josephs (1932) and Elvehjem and Sherman (1932). Cunningham concluded that the inclusion of copper in the diet has the effect of lowering the iron content of the liver and increasing that of the body. Similarly Josephs found that copper has no influence on the retention of iron but does influence the proportion of retained iron found in hemoglobin. Elvehjem and Sherman demonstrated that the addition of pure iron to the milk diet of anemic rats had no effect on the

hemoglobin content of the blood but increased the total iron content of the liver and spleen to a large extent. When the iron was fed alone for two weeks and then replaced by copper the store of iron in the liver was used for building blood hemoglobin and the iron content of the liver was reduced to a level slightly less than that in severely anemic animals. Muntwyler and Hanzal (1933) have reported a similar mobilization of stored iron due to copper feeding. When graded levels of inorganic iron were fed in the absence of copper the hemoglobin content of the blood remained unchanged and the amount of iron stored in the liver was proportional to the amount fed. In the presence of copper the rate of hemoglobin formation was dependent upon the iron intake and the liver showed no iron storage until 0.3 mgm. or more of iron was fed daily. Thus copper has no influence on iron assimilation but does function in the conversion of the iron into hemoglobin after it has been absorbed.

The hemoglobin response occurring when iron is injected intraperitoneally has brought forth some discussion concerning the relation of copper to iron assimilation. Keil and Nelson (1932) and Eveleth, Bing and Myers (1933) found very definite hemoglobin regeneration in anemic rats receiving intraperitoneal injections of pure iron. The later workers obtained complete regeneration of hemoglobin in 7 weeks with a 0.5 mgm. dosage of iron, injected on alternate days. The addition of copper to the parenterally administered iron did not accelerate the rate of hemoglobin formation significantly. In the earlier paper they point out that the rôle of copper in hemoglobin production must be evaluated in terms of iron absorption; however, in a more recent paper Bing, Saurwein and Myers (1934) suggest that the value of parenterally administered iron may also be due to an increased utilization of copper. They found that the injected rats had a much higher copper content than control rats sacrificed at the beginning of the experimental period. The higher copper content was accounted for by a greater utilization of the traces of copper in milk during the injection of iron. In a later paper, Keil and Nelson (1934a) conclude that copper may play some part in the absorption of iron, but the data show clearly that the relation of copper in hemoglobin formation cannot be explained on this basis alone.

Unpublished data from the author's laboratory show that, although there is a small amount of hemoglobin production during intraperitoneal injection of pure iron in severely anemic rats, the response does not compare with that obtained when copper is also supplied or when iron and copper are given orally. A very slight increase in the copper content of the whole rats and livers of the injected rats over those receiving iron

orally has been observed, but it is difficult to determine the exact source of this copper.

The fact that copper plays no direct rôle in the assimilation of iron makes it necessary to consider other mechanisms through which copper may function. Since very little is known about the synthesis of hematin compounds in living matter it is difficult to follow the relation of copper to hemoglobin formation. It is entirely possible that copper is concerned with the normal functioning of general body activities, and that the body's inability to form hemoglobin depends upon changes in these activities. Cohen and Elvehjem (1934) found the *a* component of cytochrome to be absent or greatly reduced in heart and liver tissue taken from severely anemic rats. Iron alone had no effect on the regeneration of this compound, but the feeding of copper alone or copper plus iron to the anemic rats for a few days greatly intensified the spectrum of the *a* component. Elvehjem (1931) found a similar effect of copper on the cytrochrome of yeast. The amount of oxidase in the liver of anemic rats as determined by the "Nadi" reagent was also greatly reduced. The feeding of copper or copper and iron markedly increased the oxidase content, but iron alone had no effect. Thus very definite changes aside from the retarded hemoglobin formation can be observed in animals receiving insufficient copper. It is impossible to determine at present whether these changes are sufficient to account for the body's inability to form hemoglobin or if these changes appear parallel with other disturbances. Locke, Rosbash and Shinn (1934) have studied the relation of copper and iron to the motivation of cellular metabolism. Methods have been developed for determining the copper and iron presumed to be directly active in cellular metabolism and attempts have been made to correlate these values with the type of metabolism in different tissues. Metabolizing tissues contain so many active components that a study of this type is extremely difficult. Cook, Haldane and Mapson (1931) have suggested that formic acid dehydrogenase may be a copper compound.

Keil and Nelson (1934) have attempted to correlate copper with carbohydrate metabolism. Administration of copper alone to anemic rats produced a different type of glucose tolerance curve than that obtained for the same animal before treatment. Pure iron had no effect. Handovsky (1933) and Lipman (1934) found that very small amounts of copper greatly accelerated glycogen decomposition and glycolysis in vitro as well as in the animal body. Slightly higher levels of copper had a very definite inhibiting action on glycolysis.

The relation of copper to cysteine and other sulphydril compounds has attracted considerable attention. The catalytic effect of salts of copper on the oxidation of cysteine was first demonstrated by Mathews and Walker (1909). Factors affecting this reaction have been studied by Harrison (1927), Warburg (1927), Elvehjem (1930), and Elliott (1930). The action of copper in the oxidation of glutathione is not so definite. Meldrum and Dixon (1930) state that pure reduced glutathione is not oxidizable in the presence of copper, but Rosenthal and Voegtlin (1931) suggest that copper can accelerate the oxidation of SH-glutathione. The interaction of copper and sulphydril compounds may play an important rôle in the regulation of the activity of certain enzyme systems. Krebs (1930) found 3×10^{-3} mgm. copper per cubic centimeter to produce an 80 per cent inhibition in the activity of papain. The inhibition was removed by addition of KCN, H_2S, cysteine and pyrophosphate. Sumner and Poland (1933), Hellerman, Perkins and Clark (1933) and Bersin and Logemann (1933) have concluded that the sulphydril group concerned with the activity of such enzymes as urease and papain is contained in the enzyme molecule itself. Michaelis and Runnström (1934) have come to the same conclusion for the glycolytic enzyme system of muscle extract. The enzyme loses its enzymatic property by oxidation to the disulphide state and is reactivated by reduction to the sulphydril state.

A somewhat similar relation between copper and ascorbic acid and the activity of certain enzymes has been suggested. This work has been reviewed by von Euler (1934). It is thus seen that quantities of copper sufficient to determine the rate of hemoglobin formation may have very definite effects on certain enzyme systems.

A discussion of the biological significance of copper would be incomplete without some mention of the possible toxic effect of copper. Everyone knows that copper, like many other essential elements, is extremely toxic in large doses. However, the large number of experimental animals which have been grown to maturity on milk mineralized with iron and copper demonstrates without doubt that copper in moderate doses produces no ill effects. Coulson, Remington and Lynch (1934) have reviewed very recently the important work on the possible chronic poisoning due to the ingestion of small amounts of copper over long periods of time. Mallory and co-workers (1925, 1931) have attempted to show a relation between copper poisoning and hemochromatosis. Sheldon (1934) in a recent review on hemochromatosis states that it is not possible to accept the view of Mallory that the disease is produced by chronic copper poisoning.

The type of diet is very important in determining the level of copper which is toxic. Waddell, Steenbock and Hart (1931) found 4 mgm. of copper as $CuSO_4$ per rat daily given in milk to be injurious, but rats given 4 mgm. of copper added to a mixed diet survived and grew normally. Similar results have been obtained by Flinn and Inouye (1929) and Coulson and co-workers (1934). The latter workers have shown that rats receiving a diet containing 527 mgm. per kilo of copper derived either from "green oysters" or $CuSO_4$ intimately mixed with the diet showed no evidences of toxicity. There was no necrosis, pigmentation or tissue proliferation in the livers of any of the animals. The rats receiving the oysters consumed about seven times the relative amount a human would consume by eating one serving every day. However, it is well to keep in mind that after any organism has received sufficient copper to meet the requirements for normal metabolism, additional amounts are of no value and may be of some harm.

CONCLUSION

In conclusion we may summarize briefly as follows: Copper is present in all living matter. The amount varies greatly in different organisms as well as in the different tissues of the same organism. Two naturally occurring copper compounds are known, hemocyanin and turacin. Copper is necessary as a supplement to iron for hemoglobin formation in red-blood animals. Of a large group of elements, copper is unique in this respect. Milk is extremely low in copper as well as iron, but a variety of natural foods will supply an adequate amount for normal nutrition. In the case of increased requirements simple inorganic copper salts together with iron salts will allow adequate hemoglobin formation. Copper is essential for plant growth, but its function, aside from its association with chlorophyll formation, is unknown. It also seems to be essential for yeast and other microörganisms. In the animal body it is not concerned with the assimilation of iron but with the transformation of the ingested iron into hemoglobin. Recent studies concerning the mechanism of copper activity in the animal body deal mainly with distribution of copper in different tissues, changes in the copper content of blood, the effect of copper on iron storage and utilization, and its relation to certain enzyme systems. A complete understanding of the action of copper will bring us distinctly nearer an understanding of the fundamental processes in living matter.

REFERENCES

ABDERHALDEN, E. AND P. MÖLLER. Ztschr. physiol. Chem. **176**: 95, 1928.

ADAMSON, J. D. AND F. H. SMITH. Can. M. A. J. **24**: 793, 1931.

ALSBERG, C. L. AND E. D. CLARK. J. Biol. Chem. **8**: 1, 1910.

ALSBERG, C. L. AND W. M. CLARK. J. Biol. Chem. **19**: 503, 1914.

ALLISON, R. V., O. C. BRYAN AND J. H. HUNTER. Fla. Agr. Exp. Sta. Bull. 190, 1927.

ANSBACHER, S., R. E. REMINGTON AND F. B. CULP. Ind. Eng. Chem., anal. ed. **3**: 314, 1931.

ATKINS, W. R. G. J. Mar. Biol. Assoc. N.S. **18**: 193, 1932.

BEARD, H. H., R. W. BAKER AND V. C. MYERS. J. Biol. Chem. **94**: 123, 1931.

BEARD, H. H. AND V. C. MYERS. J. Biol. Chem. **94**: 71, 1931.

BEGEMANN, H. 1924. Thesis, Utrecht. For abstract see H. JORDAN, Ztschr. vergl. Physiol. **2**: 381, 1925.

BERSIN, T. AND W. LOGEMANN. Ztschr. physiol. Chem. **220**: 209, 1933.

BERT, P. Compt. rend. Acad. **65**: 300, 1867.

BERTRAND, G. Bull. soc. sci. hyg. aliment. **8**: 49, 1920.

BETHELL, F. H., S. M. GOLDHAMER, R. ISAACS AND C. C. STURGIS. J. A. M. A. **103**: 797, 1934.

BIAZZO, R. Ann. Chim. Appl. **16**: 2, 1926.

BING, F. C., E. M. SAURWEIN AND V. C. MYERS. J. Biol. Chem. **105**: 343, 1934.

BLASIUS, W. Ztschr. ration. Medicine 3te Reihe. **26**: 250, 1866.

BLOXSOM, A. P. South. Med. J. **25**: 401, 1932.

BODANSKY, M. J. Biol. Chem. **48**: 361, 1921.

BORTELS, H. Biochem. Ztschr. **182**: 301, 1927.

BRAND, E. AND C. J. STUCKY. Proc. Soc. Exp. Biol. Med. **31**: 689, 1934.
Proc. Soc. Exp. Biol. Med. **31**: 627, 1934a.

BRENCHLEY, W. E. J. Agric. Sci. **22**: 704, 1932.

BRYAN, O. C. J. Am. Soc. Agron. **21**: 923, 1929.

CALDWELL, G. W. AND R. H. DENNETT. Med. J. Record **135**: 286, 1932.

CALLAN, T. AND J. A. R. HENDERSON. Analyst **54**: 650, 1929.

CASON, J. F. J. Ped. **4**: 614, 1934.

CHERBULIEZ, E. AND S. ANSBACHER. Helv. Chim. Acta **13**: 187, 1930.

CHEVREUL, M. E. Compt. rend. Acad. **66**: 567, 1868.

CHURCH, A. W. Proc. Roy. Soc., London **17**: 436, 1869.

COHN, E. J., G. R. MINOT, J. F. FULTON, H. F. ULRICHS, F. C. SARGENT, J. H. WEARE AND W. P. MURPHY. J. Biol. Chem. **74**: lxix, 1927.

COHEN, E. AND C. A. ELVEHJEM. J. Biol. Chem. **107**: 97, 1934.

CONANT, J. B., B. F. CHOW AND E. B. SCHOENBACH. J. Biol. Chem. **101**: 463, 1933.

CONANT, J. B., F. DERSCH AND W. E. MYDANS. J. Biol. Chem. **105**: 755, 1934.

CONN, L. W., A. H. JOHNSON, H. A. TREBLER AND V. KARPENKO. Ind. Eng. Chem., anal. ed. **7**: 15, 1935.

COOK, R. P., J. B. S. HALDANE AND L. W. MAPSON. Biochem. J. **25**: 534, 1931.

COULSON, E. J., R. E. REMINGTON AND K. M. LYNCH. Bur. Fish. Invest. Report no. 27, Washington, 1934.

CUNNINGHAM, I. J. Biochem. J. **25**: 1267, 1931.

DAMESHEK, W. J. A. M. A. **100**: 540, 1933.

DANIELS, A. L. AND O. E. WRIGHT. J. Nutr. **8**: 125, 1934.

DAVIDSON, L. S. P. AND I. LEITCH. Nutr. Abst. Rev. **3**: 901, 1934.
DAVIES, W. L. J. Dairy Research **3**: 86, 1931.
J. Dairy Research **4**: 255, 1933.
DESCHAMPS, M. J. pharm. chim., 3 sér. **13**: 91, 1848.
DHÉRÉ, C. J. physiol. path. gén. **16**: 985, 1915.
J. physiol. path. gén. **18**: 221, 1919.
J. physiol. path. gén. **18**: 1081, 1920.
DHÉRÉ, C. AND A. BURDEL. J. physiol. path. gén. **18**: 685, 1919.
DRABKIN, D. L. AND H. K. MILLER. J. Biol. Chem. **90**: 531, 1931.
DRABKIN, D. L. AND C. S. WAGGONER. J. Biol. Chem. **89**: 51, 1930.
DUTOIT, P. AND C. ZBINDEN. Compt. rend. **190**: 172, 1930.
DWYER, H. V. J. Michigan State Med. Soc. **29**: 420, 1930.
ELDEN, C. A., W. M. SPERRY, F. S. ROBSCHEIT-ROBBINS AND G. H. WHIPPLE. J. Biol. Chem. **79**: 577, 1928.
ELLIOTT, K. A. C. Biochem. J. **24**: 310, 1930.
ELVEHJEM, C. A. Biochem. J. **24**: 415, 1930.
J. Biol. Chem. **90**: 111, 1931.
J. A. M. A. **98**: 1047, 1932.
ELVEHJEM, C. A. AND E. B. HART. J. Biol. Chem. **82**: 473, 1929.
J. Biol. Chem. **84**: 131, 1929a.
J. Biol. Chem. **91**: 37, 1931.
J. Biol. Chem. **95**: 363, 1932.
ELVEHJEM, C. A., E. B. HART, H. C. JACKSON AND K. G. WECKEL. J. Dairy Sci. **17**: 763, 1934.
ELVEHJEM, C. A., E. B. HART AND W. C. SHERMAN. J. Biol. Chem. **103**: 61, 1933; J. Ped. **4**: 65, 1934.
ELVEHJEM, C. A. AND A. R. KEMMERER. J. Biol. Chem. **93**: 189, 1931.
ELVEHJEM, C. A., A. R. KEMMERER, E. B. HART AND J. G. HALPIN. J. Biol. Chem. **85**: 89, 1929.
ELVEHJEM, C. A. AND C. W. LINDOW. J. Biol. Chem. **81**: 435, 1929.
ELVEHJEM, C. A. AND W. C. SHERMAN. J. Biol. Chem. **98**: 309, 1932.
ELVEHJEM, C. A., H. STEENBOCK AND E. B. HART. J. Biol. Chem. **83**: 21, 1929.
J. Biol. Chem. **83**: 27, 1929a.
J. Biol. Chem. **93**: 197, 1931.
ERIKSON, S. E., R. E. BOYDEN, J. H. MARTIN AND W. M. INSKO, JR. Ky. Agric. Exp. Sta. Bull., 342, 1933.
EVELETH, M. W., F. C. BING AND V. C. MYERS. Proc. Soc. Exp. Biol. Med. **30**: 852; J. Biol. Chem. **101**: 359, 1933.
FELIX, E. L. Phytopath. **17**: 49, 1927.
FISCHER, H. AND J. HILGER. Ztschr. physiol. Chem. **128**: 167, 1923.
FISCHER, H. AND G. LEOPOLDI. Angew. Chem. **47**: 90, 1934.
FITZ-HUGH, T., JR., G. M. ROBSON AND D. L. DRABKIN. J. Biol. Chem. **103**: 617, 1933.
FLEURENT, E. AND L. LEVI. Bull. soc. Chim. **27**: 440, 1920.
FLINN, F. B. AND J. M. INOUYE. J. Biol. Chem. **84**: 101, 1929.
FOX, H. M. AND H. RAMAGE. Proc. Roy. Soc., London B **108**: 157, 1931.
FREDERICQ, L. Arc. zool. exp. et. gén. **7**: 535, 1878.
FULLERTON, H. W. Edinburgh Med. J. **41**: 99, 1934.

GEBHARDT, H. T. AND H. H. SOMMER. Ind. Eng. Chem., anal. ed. **3**: 24, 1931.
GERAGHTY, G. B., F. A. UNDERHILL, J. M. ORTEN AND R. C. LEWIS. J. Biol. Chem. **99**: 451, 1933.
GÖRTER, E., F. GRENDEL AND W. A. M. WEYERS. Rév. franc. de péd. **7**: 747, 1931.
GRENDEL, F. Pharm. Weekblad. **67**: 1345, 1930.
GRIFFITHS, A. B. Proc. Roy. Soc. Edinburgh **18**: 288, 1892.
GROSS, E. L. Clin. Med. Surg. **39**: 567, 1932.
GUERITHAULT, B. Compt. rend. Acad. **171**: 196, 1920.
Bull. soc. sci. hyg. aliment. **15**: 386, 1927.
GUILLEMET, R. Compt. rend. Soc. Biol. **108**: 32, 1931.
Compt. rend. Soc. Biol. **109**: 652, 1932.
Compt. rend. Soc. Biol. **109**: 221, 1932a.
GUILLEMET, R. AND G. GOSSELIN. Compt. rend. Soc. Biol. **111**: 41, 1932.
HAMILTON, T. S., G. E. HUNT AND W. E. CARROLL. J. Agric. Res. **47**: 543, 1933.
HANDOVSKY, H. Biochem. Ztschr. **265**: 110, 1933.
HARLESS, E. Arch. Anat. Physiol. 148, 1847.
HARRIS, R. S. Science **76**: 495, 1932.
HARRISON, D. C. Biochem. J. **21**: 335, 1927.
HART, E. B., C. A. ELVEHJEM, H. STEENBOCK, G. BOHSTEDT AND J. M. FARGO. J. Nutr. **2**: 277, 1930.
Wisconsin Agric. Exp. Sta. Bull. 409, 1929.
HART, E. B., C. A. ELVEHJEM, J. WADDELL AND R. C. HERRIN. J. Biol. Chem. **72**: 299, 1927.
HART, E. B., H. STEENBOCK, C. A. ELVEHJEM AND J. WADDELL. J. Biol. Chem. **65**: 67, 1925.
HART, E. B., H. STEENBOCK, J. WADDELL AND C. A. ELVEHJEM. J. Biol. Chem. **77**: 797, 1928.
HEATH, C. W. Arch. Internal. Med. **51**: 459, 1933.
HELLERMAN, L. H., M. E. PERKINS AND W. M. CLARK. Proc. Nat. Acad. Sci. Washington, D. C., **19**: 855, 1933.
HENZE, M. Ztschr. physiol. Chem. **33**: 370, 1901.
Ztschr. physiol. Chem. **72**: 494, 1911.
Ztschr. physiol. Chem. **79**: 215, 1912.
HERKEL, W. Beitr. path. Anat. **85**: 513, 1930.
HESS, A. F., G. C. SUPPLEE AND B. BELLIS. J. Biol. Chem. **57**: 725, 1923.
HESS, A. F. AND L. J. UNGER. Proc. Soc. Exp. Biol. Med. **19**: 119, 1921–22.
HILTNER, R. S. AND H. J. WICHMAN. J. Biol. Chem. **38**: 205, 1919.
HOAGLAND, D. R. Annual Rev. Biochem. **1**: 618, 1932.
HODGES, M. A. AND W. H. PETERSON. J. Am. Diet. Assoc. **7**: 6, 1931.
HOGBEN, L. T. AND K. F. PINHEY. Brit. J. Exp. Biol. **4**: 203, 1926.
Brit. J. Exp. Biol. **5**: 55, 1927.
JOLYET, F. AND P. REGNARD. Arch. physiol. norm. et. path. **4**: 584, 1877.
JOSEPHS, H. W. Bull. Johns Hopkins Hosp. **49**: 246, 1931.
J. Biol. Chem. **96**: 559, 1932.
KEIL, H. AND V. E. NELSON. Proc. Soc. Exp. Biol. Med. **28**: 392, 1931.
J. Biol. Chem. **93**: 49, 1931a.
J. Biol. Chem. **97**: 115, 1932.

KEIL, H. AND V. E. NELSON. J. Biol. Chem. **106**: 343, 1934.
J. Lab. Clin. Med. **19**: 1083, 1934a.
J. Pediat. **19**: 1081, 1934b.
KEILIN, D. Proc. Roy. Soc. London B. **100**: 129, 1926.
KEMMERER, A. R., C. A. ELVEHJEM AND E. B. HART. J. Biol. Chem. **92**: 623, 1931.
KEMMERER, A. R., C. A. ELVEHJEM, E. B. HART AND J. M. FARGO. Am. J. Physiol. **102**: 319, 1932.
KING, C. G. AND G. ETZEL. Ind. Eng. Chem. **19**: 1004, 1927.
KLEINMANN, H. AND J. KLINKE. Arch. path. Anat. Physiol. **275**: 422, 1930.
KONDO, K. AND S. MORI. J. Chem. Soc., Japan, **53**: 1190, 1932.
KOSAKA, S. Aichi. Igakkai. Zassi. **38**: 2498, 1931.
KRAUSS, W. E. J. Dairy Sci. **12**: 242, 1929.
J. Biol. Chem. **90**: 267, 1931.
KREBS, H. A. Klin. Wchnschr. **7**: 584, 1928.
Biochem. Ztschr. **220**: 289, 1930.
LAIDLAW, P. P. J. Physiol. **31**: 464, 1904.
LEHMANN, K. B. Arch. Hyg. **24**: 18, 1895.
LEWIS, G. T., T. E. WEICHSELBAUM AND J. L. MCGHEE. Proc. Soc. Exp. Biol. Med. **27**: 329, 1930.
LEWIS, M. S. J. A. M. A. **96**: 1135, 1931.
LIEGEOIS, M. Bull L'Acad. Med. 45: 492, 1901.
LINDOW, C. W., C. A. ELVEHJEM AND W. H. PETERSON. J. Biol. Chem. **82**: 465, 1929.
LINDOW, C. W., W. H. PETERSON AND H. STEENBOCK. J. Biol. Chem. **84**: 419, 1929.
LIPMAN, F. Biochem. Ztschr. **268**: 314, 1934.
LIPMAN, C. B. AND G. MACKINNEY. Plant Physiol. **6**: 563, 1931.
LOCKE, A., E. R. MAIN AND D. O. ROSBASH. J. Clin. Invest. **11**: 527, 1932.
LOCKE, A., D. O. ROSBASH AND L. E. SHINN. J. Infect. Dis. **54**: 51, 1934.
LOESCHKE, A. Ztschr. physiol. Chem. **199**: 125, 1931.
MACHOLD, K. Ztschr. klin. Med. **127**: 27, 1934.
MACKAY, H. M. M. Arch. Dis. Child. **8**: 145, 1933.
MALLORY, F. B. Am. J. Path. **1**: 117, 1925.
MALLORY, F. B. AND F. PARKER, JR. Am. J. Path. **7**: 351, 1931.
MAQUENNE, L. AND E. DEMOUSSY. Compt. rend. Acad. **170**: 87, 1920.
MATHEWS, A. P. AND S. WALKER. J. Biol. Chem. **6**: 299, 1909.
MCFARLANE, W. D. Biochem. J. **26**: 1022, 1932.
MCFARLANE, W. D. AND H. I. MILNE. J. Biol. Chem. **107**: 309, 1934.
MCHARGUE, J. S. Am. J. Physiol. **72**: 583, 1925.
Am. J. Physiol. **77**: 245, 1926.
MCHARGUE, J. S. AND R. K. CALFEE. Plant Physiol. **6**: 559, 1931.
MCHARGUE, J. S., D. J. HEALY AND E. S. HILL. J. Biol. Chem. **78**: 637, 1928.
MEISSNER, W. Ann. chim. phys. **4**: 106, 1817.
MELDRUM, N. U. AND M. DIXON. Biochem. J. **24**: 472, 1930.
MENDEL, L. B. AND H. C. BRADLEY. Am. J. Physiol. **17**: 167, 1907.
MEYER, A. E. AND C. EGGERT. J. Biol. Chem. **99**: 265, 1932.
MICHAELIS, L. AND J. RUNNSTRÖM. Proc. Soc. Exp. Biol. Med. **32**: 343, 1934.
MIDDLETON, W. S. J. A. M. A. **91**: 857, 1928.
MILLS, E. S. Can. M. A. J. **22**: 175, 1930.
Am. J. Med. Sci. **182**: 554, 1931.

MILLER, L. AND H. S. MITCHELL. J. Am. Dietetic Assoc. **7**: 252, 1931.
MITCHELL, H. S. AND M. VAUGHN. J. Biol. Chem. **75**: 123, 1927.
MITCHELL, H. S. AND L. MILLER. J. Biol. Chem. **85**: 355, 1929–30.
J. Biol. Chem. **92**: 421, 1931.
MORRISON, D. B. AND T. P. NASH, JR. J. Biol. Chem. **88**: 479, 1930.
MUNTWYLER, E. AND R. F. HANZAL. Proc. Soc. Exp. Biol. Med. **30**: 845, 1933.
MYERS, V. C. AND H. H. BEARD. J. A. M. A. **93**: 1210, 1929.
MYERS, V. C., D. G. REMP AND F. C. BING. Abstract Division of Biological Chemistry, A. C. S., Cleveland, Sept. 10–14, 1934.
NEAL, W. M. AND R. B. BECKER. J. Agric. Res. **47**: 249, 1933.
ORENT, E. R. AND E. V. MCCOLLUM. J. Biol. Chem. **92**: 651, 1931.
ORTEN, J. M., F. A. UNDERHILL AND R. C. LEWIS. J. Biol. Chem. **96**: 1, 1932.
ORTH, O. S., G. C. WICKWIRE AND W. E. BURGE. Science **79**: 34, 1934.
PARSONS, L. G. AND J. C. HAWKSLEY. Arch. Dis. Child. **8**: 117, 1933.
PARSONS, L. G. AND E. M. HICKMANS. Arch. Dis. Child. **8**: 95, 1933.
PARSONS, T. R. AND W. PARSONS. J. Gen. Physiol. **6**: 153, 1923.
PREGL, F. Quantitative organic microanalysis. Transl. by E. Fyleman, London, 2nd ed., 1924.
QUAGLIARIELLO, G. D. Mollusken in Winterstein, H. Handbuch der Vergleichenden Physiologie **1**: 597. Jena, 1924.
QUAM, G. N. AND A. HELLWIG. J. Biol. Chem. **78**: 681, 1928.
QUARTAROLI, A. Ann. chim. appl. **18**: 47, 1928.
QUINQUAUD, M. Compt. rend. Acad. **77**: 487, 1873.
RABINOWITCH, I. M. J. Biol. Chem. **100**: 479, 1933.
RAMAGE, H., J. H. SHELDON AND W. SHELDON. Proc. Roy. Soc., London B. **113**: 308, 1933.
REDFIELD, A. C. Biol. Rev. **9**: 175, 1934.
REDFIELD, A. C., T. COOLIDGE AND A. L. HURD. J. Biol. Chem. **69**: 475, 1926.
REDFIELD, A. C., T. COOLIDGE AND H. MONTGOMERY. J. Biol. Chem. **76**: 197, 1928.
REDFIELD, A. C. AND D. R. GOODKIND. Brit. J. Exp. Biol. **6**: 340, 1929.
REDFIELD, A. C., T. COOLIDGE AND M. A. SHOTTS. J. Biol. Chem. **76**: 185, 1928.
REMINGTON, R. E. Science **77**: 115, 1933.
REMINGTON, R. E. AND H. E. SHIVER. J. A. O. A. C. **13**: 129, 1930.
REMY, E. Z. Untersuch. Lebensm. **64**: 545, 1932.
RIDER, T. H. J. Biol. Chem. **100**: 243, 1933.
ROBSCHEIT-ROBBINS, F. S., C. A. ELDEN, W. M. SPERRY AND G. H. WHIPPLE. J. Biol. Chem. **79**: 563, 1928.
RONCATO, A. AND B. BASSANI. Arch. sci. biol. **19**: 541, 1934.
ROSE, W. C. AND M. BODANSKY. J. Biol. Chem. **44**: 99, 1920.
ROSENTHAL, S. M. AND C. VOEGTLIN. Pub. Health Reports **46**: 521, 1931.
SANDBERG, M. AND O. M. HOLLEY. Proc. Soc. Exp. Biol. Med. **32**: 307, 1934.
SANDBERG, M. AND D. PERLA. J. Exp. Med. **60**: 395, 1934.
SARATA, U. Japan J. Med. Sci. II. Biochem. **2**: 247, 1933.
Japan J. Med. Sci. II. Biochem. **2**: 261, 1933a.
Japan J. Med. Sci. II. Biochem. **2**: 309, 1933b.
SARATA, U. AND A. SUZUKI. Japan J. Med. Sci. II. Biochem. **2**: 341, 1933.
SARZEAU, M. J. pharm. et chim. **16**: 505, 1830.

SCHÖNHEIMER, R. AND F. OSHIMA. Ztschr. physiol. Chem. **180**: 249, 1929.
SCHUETTE, H. A. AND K. REMY. J. Am. Chem. Soc. **54**: 2909, 1932.
SCHULTZE, K. W. Klin. Wchnschr. **11**: 497, 1932.
SCHULTZE, M. O. AND C. A. ELVEHJEM. J. Biol. Chem. **102**: 357, 1933.
SCHULTZE, M. O., C. A. ELVEHJEM AND E. B. HART. J. Biol. Chem. **106**: 735, 1934.
SEVERY, H. W. J. Biol. Chem. **55**: 79, 1923.
SHELDON, J. H. Brit. Med. J. **2**: 869, 1932.
Lancet, November 10, 1031, 1934.
SHELDON, J. H. AND H. RAMAGE. Biochem. J. **25**: 1608, 1931.
SHERMAN, W. C., C. A. ELVEHJEM AND E. B. HART. J. Biol. Chem. **107**: 289, 1934.
SHINDEL, L. Klin. Wchnschr. **10**: 743, 1931.
SJOLLEMA, B. Biochem. Ztschr. **267**: 151, 1933.
SKINNER, J. T., W. H. PETERSON AND H. STEENBOCK. Biochem. Ztschr. **150**: 392, 1932.
SKINNER, J. T., H. STEENBOCK AND W. H. PETERSON. J. Biol. Chem. **97**: 227, 1932.
SOMMER, A. L. Plant Physiol. **6**: 339, 1931.
SPACU, G. Analyst **49**: 275, 1924.
STEDMAN, E. AND E. STEDMAN. Biochem. J. **19**: 544, 1925.
Biochem. J. **20**: 938, 1926.
STEIN, H. B. AND R. C. LEWIS. J. Nutr. **6**: 465, 1933.
SUMNER, J. B. AND L. O. POLAND. Proc. Soc. Exp. Biol. and Med. **30**: 553, 1933.
SUPPLEE, G. C. AND B. BELLIS. J. Dairy Sci. **5**: 455, 1922.
SUZUKI, A. AND U. SARATA. Japan J. Med. Sci. II. Biochem. **2**: 309, 1933.
THOMAS, B. H. AND S. S. WHEELER. Am. Soc. Anim. Prod. Proc. **24**: 204, 1933.
THUDICUM, J. L. W. Die Chemische Constitutions des Gehirns des Menschen und der Tiere. Tübingen, 1901.
TITUS, R. W., H. W. CAVE AND J. S. HUGHES. J. Biol. Chem. **80**: 565, 1929.
TOMPSETT, S. L. Biochem. J. **28**: 1544, 1934.
UNDERHILL, F. A., J. M. ORTEN, E. R. MUGRAGE AND R. C. LEWIS. J. Biol. Chem. **99**: 469, 1933.
VON EULER, H. Ergebnisse der Enzymforschung III. Leipzig, 157, 1934.
WADDELL, J., H. STEENBOCK AND E. B. HART. J. Biol. Chem. **83**: 243, 1929.
J. Biol. Chem. **84**: 115, 1929a.
J. Nutr. **4**: 53, 1931.
WARBURG, O. Biochem. Ztschr. **187**: 255, 1927.
WARBURG, O. AND H. A. KREBS. Biochem. Ztschr. **190**: 143, 1927.
WAUGH, T. R. Arch. Int. Med. **47**: 71, 1931.
WEBSTER, E. J. AND F. JANSMA. Science **70**: 174, 1929.
WHIPPLE, G. H. How the body builds its hemoglobin and blood plasma proteins. Joint Session of the Federation of American Societies for Experimental Biology, New York City, 1934.
WHIPPLE, G. H. AND F. S. ROBSCHEIT-ROBBINS. Series of papers in Am. J. Physiol. **72, 79, 80, 83**: 1925–27.
WILKERSON, V. A. J. Biol. Chem. **104**: 541, 1934.
WILLARD, J. T. J. Am. Chem. Soc. **30**: 902, 1908.
WINTROBE, M. M. AND R. T. BEEBE. Medicine **12**: 187, 1933.
WOLFF, L. K. AND A. EMMERIE. Biochem. Ztschr. **228**: 443, 1930.
ZONDEK, S. G. AND M. BANDMANN. Klin. Wchnschr. **10**: 1528, 1931.